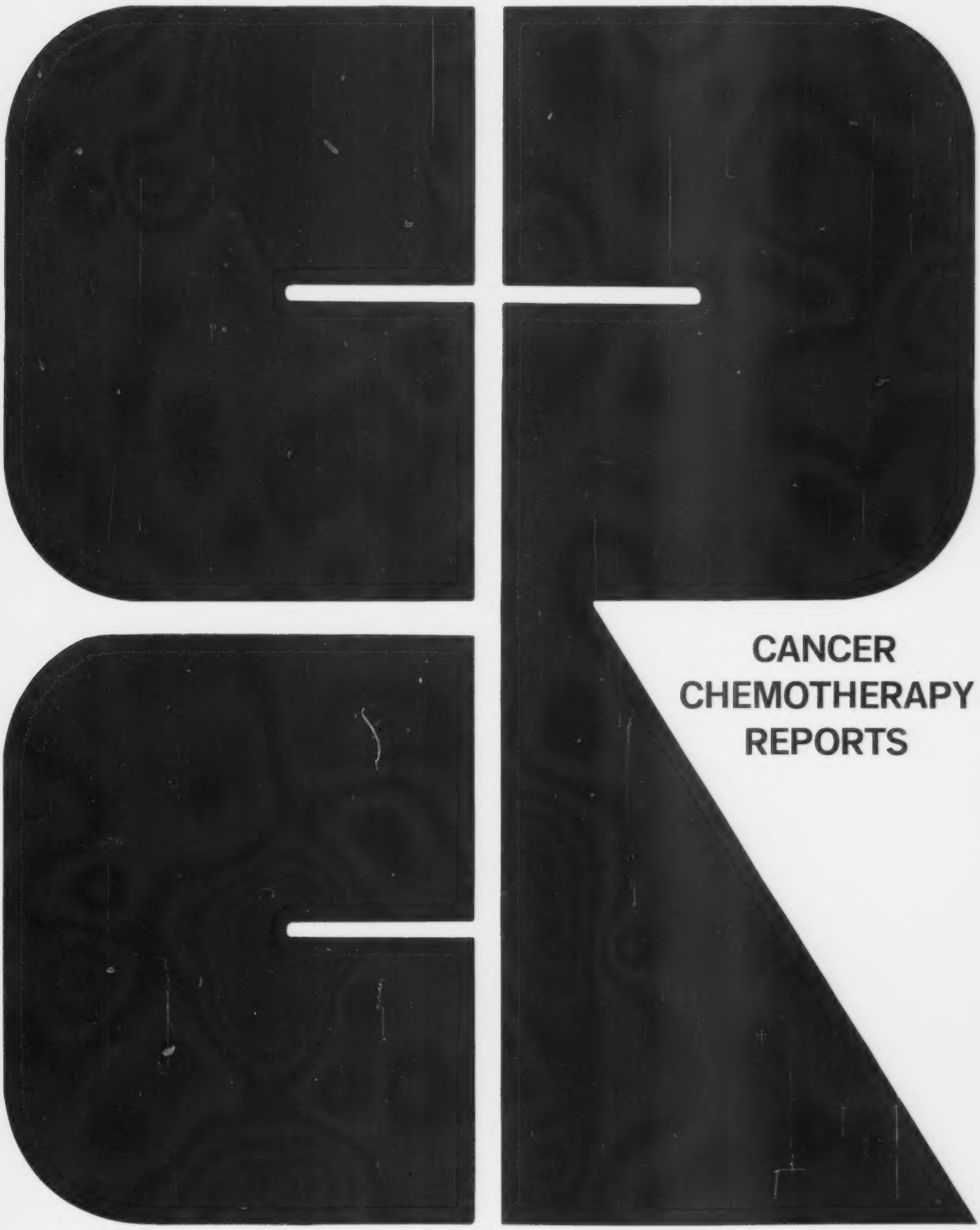

CANCER CHEMOTHERAPY REPORTS

Part 1

Jan/Feb 1974
Vol. 58, No. 1

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National Cancer Institute

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Selman A. Waksman Conference on Actinomycins:
Their Potential for Cancer Chemotherapy

Children's Cancer Research Foundation

Sidney Farber, MD, presiding

Johannes Meienhofer and Emil Frei III,
Conference Chairmen and Editors

Boston, Massachusetts

February 5 and 6, 1973

This conference on actinomycin was named by Dr. Sidney Farber to honor the discoverer of actinomycin, Dr. S. A. Waksman. With deep regret we saw both men pass away shortly after the conference was held. Dr. Farber died March 30, 1973, and Dr. Waksman died August 16, 1973. The efforts of these two distinguished scientists in making actinomycin of benefit to mankind should be taken as a mandate to continue their work.

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The Secretary of Health, Education, and Welfare has determined that the publication of this periodical is necessary in the transaction of the public business required by law of this Department. Use of funds for printing this periodical has been approved by the Director of the Office of Management and Budget through November 30, 1973.

Preface

Johannes Meienhofer [1] and Emil Frei III [2]

Actinomycin, discovered by Waksman and Woodruff (1) in 1940 and first given to patients by Schulte (2,3) in 1952, is one of the longer known cancer chemotherapeutic agents. However, an apparent narrow range of clinical usefulness and the development of many other antitumor agents have limited the clinical use of this agent. On the other hand, actinomycin became one of the most widely used biochemical tools in molecular biology because of its selective inhibition of DNA-primed RNA synthesis. The history of actinomycin represents, indeed, a curious sequence of paradoxes. Actinomycin is one of the most potent cytotoxic agents, yet the nature of its acute toxicity in animals is not understood (4); the main molecular mechanism of action of actinomycin is known, ie, inhibition of DNA-dependent RNA synthesis (5–7), yet cell death cannot be explained by this effect (8). Actinomycin is used clinically, yet clinical pharmacologic studies have never been done. An impressive amount of chemical work has been carried out, mainly by Brockmann and collaborators (9), including structure elucidation (fig 1), synthesis, and preparation of more than 100 derivatives and analogs, yet testing of these compounds for antitumor activity has been very scanty (10). Beautiful large crystals of actinomycin have been available since 1940, yet the compound has resisted X-ray crystallographic analysis until recently when Sobell et al (11) employed cocrystallization with deoxyguanosine. These are a few of the reasons that stimulated this meeting on actinomycin.

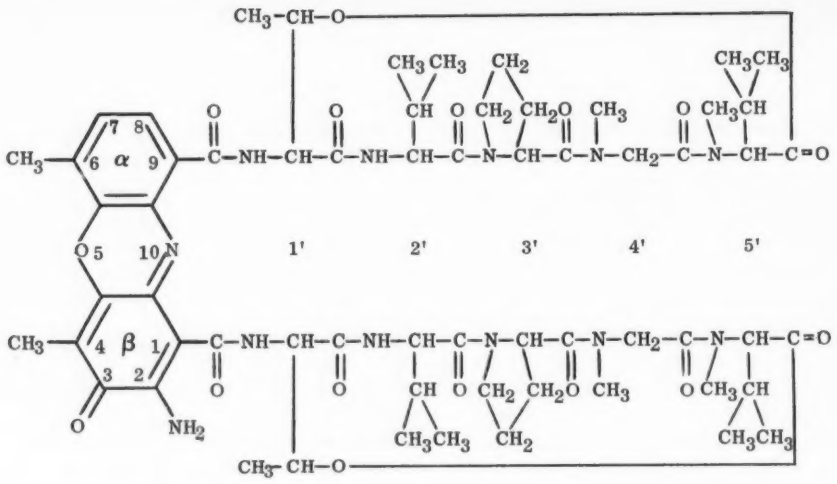
[1] Children's Cancer Research Foundation, and Department of Biological Chemistry, Harvard Medical School, Boston, Mass.

[2] Children's Cancer Research Foundation, and Department of Medicine, Harvard Medical School, Boston, Mass.

Eight years ago, Dr. Sidney Farber initiated a program of actinomycin analog synthesis at the Children's Cancer Research Foundation in a search for compounds with improved chemotherapeutic properties. We felt it was time to evaluate the results of this program in the context of the overall potential of actinomycins for cancer chemotherapy. A selected group of investigators, representing organic chemical, biochemical, pharmacologic, and clinical disciplines, were invited to attend this Conference. The response was enthusiastic, confirming the need for such a meeting.

The presentations and discussions were lively and stimulating. It was pointed out that actinomycin is one of the few agents that can produce cures in two different cancers (Wilms' tumor in children and gestational choriocarcinoma in women), and it deserves, therefore, more attention and a thorough evaluation of its therapeutic potential in several other cancers. Pharmacologic and toxicologic studies in the past were sometimes done with mixtures or insufficiently characterized samples, and, therefore, re-examination with modern screening and testing techniques are indicated, with extension of such studies to prototypes of the many derivatives and analogs that have been chemically synthesized or prepared by controlled biosynthesis (12).

The meeting has served to review the actinomycins and to indicate areas where therapeutic research might be profitably undertaken. It is hoped that this publication will stimulate renewed interest in actinomycin. We want to thank the authors for their cooperation in preparing their manuscripts on short notice and the National Cancer Institute for generously sponsoring this meeting.



[Thr—D-Val—Pro—Sar—MeVal]

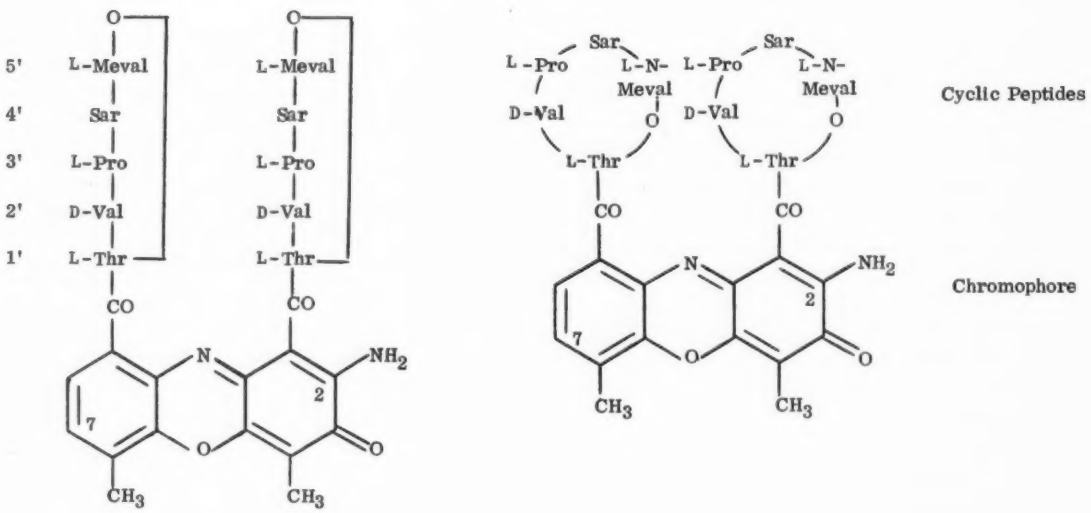


FIGURE 1.—Structure of actinomycin D (C_1) and abbreviated representations. In actinomycin C_3 both D-valine residues are replaced by D-*allo*-isoleucine residues.

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Opening Remarks

Sidney Farber [1,2]

It is a great pleasure for me to welcome you to the Selman A. Waksman Conference on Actinomycins. We are delighted that you have come to exchange knowledge, information, and ideas with one another, on what we think is a very important chapter in the field of cancer chemotherapy.

I want to thank Dr. J. Meienhofer for having been the "spark-plug" in arranging this Conference, and Drs. E. Frei III and G. E. Foley for their co-operation and help. We are very grateful to Dr. C. Gordon Zubrod and the National Cancer Institute for making it possible for us to meet here today, and to Dr. Seymour Perry who is the official representative of the National Cancer Institute.

I thought it might be worthwhile to speak of the interest of the Children's Cancer Research Foundation in the actinomycins—an interest which came about in the early 1950s as a renewal of the interest which had waned since 1940 when Drs. S. A. Waksman and H. B. Woodruff first described the isolation of actinomycin.

In 1952, C. Hackmann, working in Domagk's laboratory, described some animal tumor experiments in which an antitumor effect was produced by what he called actinomycin C. This was followed by the pioneering work of Professor Hans Brockmann, who we are honored to have with us today. Professor Brockmann's studies on the structure and synthesis of actinomycin C opened this field to other chemists and biologists.

In 1954, Dr. Waksman was good enough to give me 300 mg of actinomycin D (NSC-3053). I will never forget his arrival here, with that little vial in his hand. He delivered it himself and we started work immediately. To our great pleasure we quickly found, in 1954 and 1955, that this was the most powerful antitumor agent by weight with which we had ever worked. At that time, we were doing extensive animal work, and we had a great deal of experience with a number of compounds which exhibited antitumor activity. Toxicologic studies, and what pharmacologic studies we were able to do, were quickly carried out, and within a few months we used this new compound, whose mechanism of action was unknown, in the treatment of a child who was dying of Wilms' tumor with multiple metastases. To our great surprise and delight, this child, within a few weeks after the initiation of actinomycin D therapy, responded, as determined by roentgenographic studies of the lungs. Unfortunately, however, the disease was so widespread that she died within 3 weeks after we started treatment. There was complete disappearance of the tumor in the lung, and in its place was a hyaline, almost caseous material with no evidence of viable tumor. This gave us the impetus to conduct studies in all kinds of patients with tumors for whom we had no other therapy to offer. It was quickly clear that in addition to Wilms' tumor, Ewing's tumor responded in varying degrees to actinomycin D therapy. A few years later, in 1965, Hertz and collaborators as well as Li (in 1971) obtained striking curative effects with actinomycin D therapy alone (or in combination with the previously used methotrexate [3]) in 70%–90% of patients with gestational choriocarcinoma, even after pulmonary metastases have been found.

Figure 1 shows another patient with a Wilms' tumor that had obviously been neglected. The patient came to us late in the course of the disease. This case illustrates not only the antitumor action of actinomycin D, but the principle of interdisciplinary attack on the problem.

---

[1] Dr. Farber died Mar 30, 1973.

[2] President and Chief Executive Officer, The Children's Cancer Research Foundation; and S. B. Wolbach Professor of Pathology, Emeritus, Harvard Medical School, Boston, Mass.

[3] Methotrexate: NSC-740; CAS reg. No. 59-05-2; glutamic acid, *N*-[*p*-[[(2,4-diamino-6-pteridinyl)methyl]methylamino]benzoyl]-.

Radiotherapy was used to shrink the tumor. The tumor was removed surgically with excellent results, but at the time the child was first seen there were pulmonary metastases all through the lungs. This was a sign of an incurable tumor prior to 1954. We had never seen one disappear spontaneously, nor was it possible to give enough radiotherapy to kill the tumor without killing the lung. However, after adding actinomycin D therapy to a small amount of radiotherapy (insufficient in itself to cause disappearance of the tumor), the tumor rapidly disappeared, and almost 9 years later the child is alive (fig 1, *right*)—a result which would have been totally impossible without this new therapy. Actinomycin D therapy was continued in regular courses for 2 years, and now, 15 years after his initial chemotherapy, the patient is a big strapping boy in a state of health indistinguishable from normal.

It was the addition of actinomycin D therapy that accounted for the striking success in the treatment of this and, subsequently, many other patients with Wilms' tumors. Dr. Frei, who, I am happy to tell you, is now Physician-in-Chief of the Children's Cancer Research Foundation, will tell you about the clinical state of affairs today.

There are one or two more remarks I would like to make. In the Ciba Symposium on Peptide Chemistry, chaired by Dr. Franz Bergel in 1958, in London, there were two papers on actinomycin D. The first one was by Dr. A. W. Johnson of Nottingham, England, in which he detailed the work of Dr. E. Bullock and himself, after describing Professor Brockmann's work with actinomycin C and other actinomycins. He gave us the structure of actinomycin D, and concluded that the chemical problem was finished; he had no more interest in actinomycin D since it had no use. After I presented findings of the kind that I have indicated here, I urged him to stay with the chemical problem, because actinomycin D was just beginning to get interesting, biologically and clinically.

Here in our Foundation, members of the staff have studied various aspects of the biologic activity of actinomycin D. Dr. Foley and his colleagues have published studies on its biologic activity in bacterial systems and its mechanism of action in mammalian cell cultures. Drs. G. J. D'Angio and C. L. Maddock have studied and evaluated the synergistic effect (to use a general, rather than a specific term) between small amounts of radiation and actinomycin D therapy, or the action of actinomycin D in greatly enhancing the therapeutic effect of small amounts of radiation.

Further, we decided that we would have other members of the staff cooperate in this problem. We knew that Professor Brockmann was continuing his pioneering studies, but we did not know how much clinical assistance or biologic cooperation he was receiving. However, we decided that here was a substance with a known structure that was so interesting, both clinically and biologically, that it would be worthwhile to synthesize the drug and determine if the structure could be so altered as to wipe out its toxicity (speaking naively for a moment) and enhance its antitumor activity to improve its therapeutic index. If so, we would have a most useful agent. With this in mind, we enlisted the assistance of Dr. C. H. Li, who is with us today, and with his help, we selected Dr. Meienhofer to head a new Laboratory of Protein and Peptide Chemistry. Dr. Meienhofer came to our Foundation a little more than 8 years ago with the simple request from us that he synthesize actinomycin D. He accomplished this, with the assistance of his colleagues, after 5 years of very hard work, and he is going to tell you about the kind of chemical work he has been doing and with what purpose. In addition, Dr. E. J. Modest and his colleagues in the Laboratories of Organic Chemistry, have been working with the actinomycins, in part for another reason which you will hear about later.

I may have failed to mention other members of the staff who have been engaged in studies with actinomycin D: if so, I apologize. All in all this has been a matter of great interest in our Foundation. Thus, we are particularly pleased that you were willing to come today for a Conference which is designed to rekindle the enthusiasm and interest in what we believe is an extremely important field. Your conclusions, which Dr. Perry will summarize, may be quite to the contrary of my hopeful expectations; but even so, we will have seen and discussed the facts of the situation.

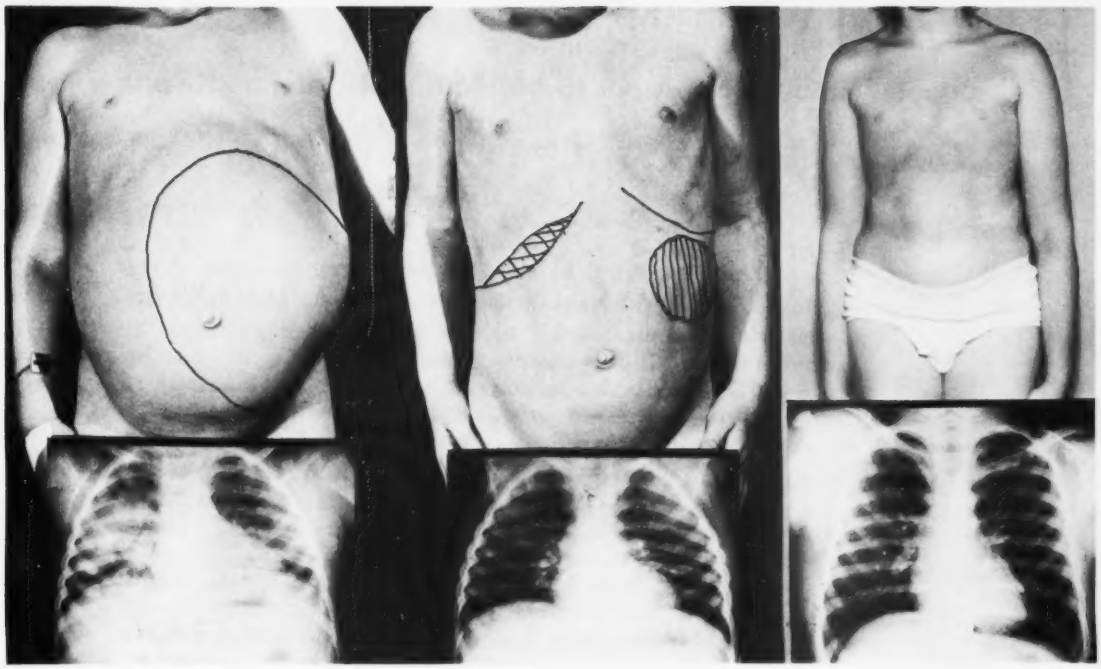


FIGURE 1.—This patient was 2 years 9 months old on admission. *Left*—Roentgenogram taken Jan 31, 1958. Photograph taken Feb 3 shows outline of large Wilms' tumor involving left kidney and multiple metastases in both lungs. Radiotherapy (3000 R) was administered to kidney region, and actinomycin D in a total dose of 75 μg/kg was administered intravenously. During the same period both lungs were radiated (1200 R). *Middle*—Feb 18, 1958. Note marked shrinkage of the Wilms' tumor and disappearance of pulmonary metastases 15 days after start of therapy. At this time nephrectomy was performed. *Right*—Almost 9 years later, Sept 16, 1966, at age 11 years 5 months. There is no evidence of metastases in lung by roentgenogram. The boy is in excellent health and is normal for his age in growth and development. [Reproduced with permission from *JAMA* (198:826–836, 1966). Copyright 1966, American Medical Association.]

History and Chemistry: Modification of the Actinomycin Molecule

Hans Brockmann [1]

SUMMARY

In the search for actinomycins with improved antineoplastic activity, derivatives and analogs have been prepared by modification of naturally occurring actinomycins and by total chemical synthesis. Reduction of the 4-oxoproline-containing actinomycin X_2 by aluminum isopropylate afforded the 4-*trans*-hydroxyproline-containing analog $X_{o\beta}$, whereas catalytic hydrogenation produced the 4-*cis*- or *allo*-hydroxyproline-containing actinomycin $X_{o\delta}$. The hydroxyl groups of both analogs have been esterified by various acids resulting in derivatives with greater affinity for water or lipids. Modifications of the actinomycin chromophore have included mono- and di-substitution at the 2-amino group, which produced mostly inactive derivatives, and substitution at the 7-carbon atom, which afforded highly active 7-nitro-actinomycin C_3 and the less active 7-amino and 7-hydroxy derivatives. However, total chemical synthesis has offered greater flexibility in the design of actinomycin analogs. The cyclization of the peptide moieties to form tightly packed pentapeptide lactones has been a key reaction. The following synthetic pathways have been developed: (*a*) cyclization of bis-*seco*-actinomycin by peptide bond formation between sarcosine and *N*-methylvaline residues, (*b*) lactonization of intermediate actinomycinic acid, and (*c*) lactonization of 2-nitro-3-benzyloxy-4-methyl-benzoyl-pentapeptides by acetyl chloride-acetylimidazole. Various routes have been used for the preparation of the required protected peptide intermediates leading to total syntheses of actinomycin C_1 (D), actinomycin C_3, and *aniso*-actinomycin C_2 (which possesses two different pentapeptide moieties with D-valine and D-*allo*-isoleucine in the 2-positions, respectively). Several peptide analogs of actinomycin C_1 (D) have been synthesized and their antibacterial potencies and constants for binding to isolated DNA have been compared. Synthetic analogs have included (*a*) those in which threonine had been replaced by serine, (*b*) those in which D-valine had been replaced by D-alanine or D-leucine, and (*c*) an enantiomer in which all eight asymmetric carbon atoms were inverted. Also synthesized were several analogs in which the two methyl groups in positions 4 and 6 of the chromophore were replaced by hydrogen, methyloxy, ethyl, and *tert*-butyl groups. Comparison of the antibacterial activities of these analogs indicates that the methyl groups are optimal for biologic activity.

[Cancer Chemother Rep 58:9–20, 1974]

[1] Organisch-Chemisches Institute der Universität Göttingen, Göttingen, Germany.

The aim of the investigations discussed in this paper was to find actinomycin derivatives or structural analogs with better therapeutic indices of anticancer action. Initially, when methods for the total synthesis of actinomycins were not available, all experiments designed to modify the chromophore or the peptide groups had to start with an actinomycin of natural origin. Therefore, experiments aimed at modifying the peptide parts of the actinomycins were restricted to the X-actinomycins that may contain hydroxyproline, a residue that may be esterified.

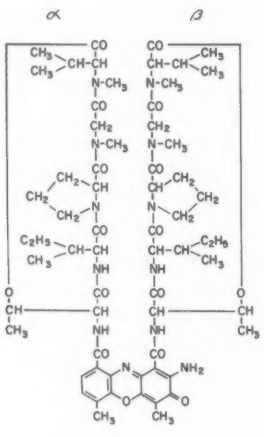
All structural and configurational modifications of the peptide rings of actinomycins and actinomycin analogs can be treated as variations of the peptide part of actinomycin C_3 (formula **1**). This was the first actinomycin for which the structure was established by both degradation and total synthesis (1). To demonstrate our modifications of the peptide part, we use simplified formulas (such as formula **3** in which the "actinocin" chromophore moiety [formula **2**] of actinomycin C_3 is symbolized by a horizontal line with two short diagonal dashes representing the amino and oxo groups of "actinocin").

Actinomycin X_2 (formula **4**), in contrast to actinomycin C_1 (D) (formula **5**), contains 4-oxoproline instead of proline in the β-peptide ring. The carbonyl oxygen of the 4-oxoproline residue may be replaced by hydrogen if actinomycin X_2 is hydrogenated by means of a platinum catalyst in acetic acid. In this way, actinomycin X_2 may be converted into actinomycin C_1 (D). This conversion was the first in vitro transformation of one actinomycin into another (2).

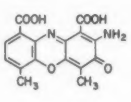
More interesting to us was the observation that under mild conditions the oxoproline residue of actinomycin X_2 may be reduced to a hydroxyproline residue. Aluminum isopropylate reduces actinomycin X_2 to actinomycin $X_{0\beta}$ (formula **6**), whereas hydrogenation in the presence of a platinum catalyst and methanol leads to actinomycin $X_{0\delta}$ (formula **7**) (2). Actinomycins $X_{0\beta}$ and $X_{0\delta}$ are synthesized in small amounts by *Streptomyces* strains that produce actinomycin X_2 as the main actinomycin (3,4).

The activities of actinomycins $X_{0\beta}$ and $X_{0\delta}$ against *Bacillus subtilis* are about 4 and 60, respectively, in comparison with the activity of actinomycin C_1 (D) (taken as 100). The influence of the configuration is remarkable. Actinomycin $X_{0\delta}$, which differs from actinomycin $X_{0\beta}$ only in the configuration of carbon atom 4 of the hydroxyproline residue (ie, the hydroxyl group in the *cis*- position), is approximately 15 times more potent against bacteria than actinomycin $X_{0\beta}$.

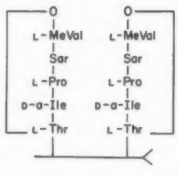
As already mentioned, the hydroxyl group



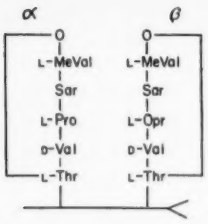
ACTINOMYCIN C_3
(1)



ACTINOCIN
(2)

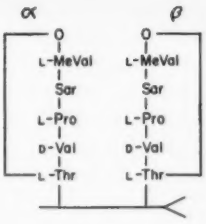


ACTINOMYCIN C_3
(3)



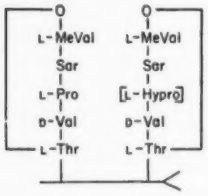
ACTINOMYCIN X_2

(4)



ACTINOMYCIN C_1(D)

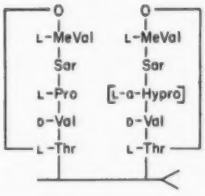
(5)



ACTINOMYCIN Xoβ

Activity : 4

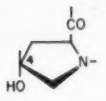
(6)



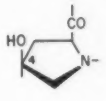
ACTINOMYCIN Xoδ

Activity : 60

(7)



L-Hypro



L-a-Hypro

Instead of -OH:

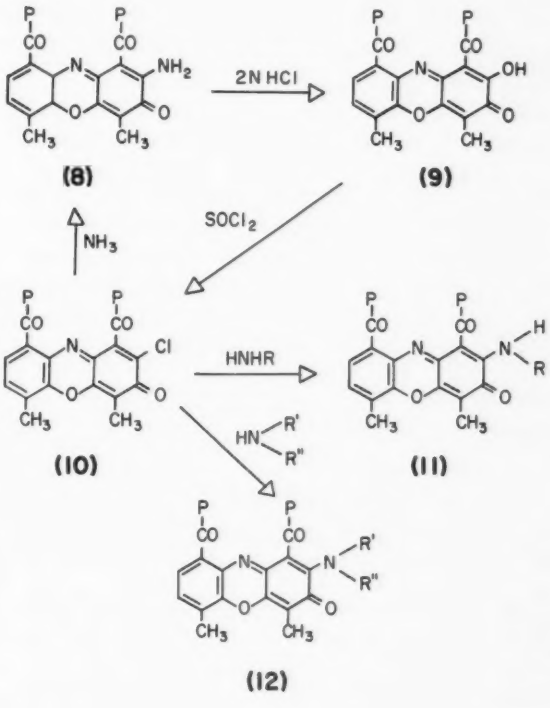
a : $-OOC-(CH_2)_{14}-CH_3$

b : $-OOC-(CH_2)_n-COOH$

c : $-OPO(OH)_2$

of the hydroxyproline residue allows alteration of the β-peptide ring of the two actinomycins $X_{o\beta}$ and $X_{o\delta}$ by means of esterification. Because actinomycin $X_{o\delta}$ has greater biologic activity, we investigated its esters only. Esterification with palmitic acid gave a crystalline ester (formula **7a**) that had greatly enhanced solubility in lipophilic solvents but only low antibiotic activity (2). Esterification with dicarbonic acid or phosphoric acid afforded acid esters such as compounds **7b**[2] and **7c** (5) that have a greater solubility in water than the parent actinomycins but only low antibiotic activities. It is probable that the activity is due to partial in vivo hydrolysis of the esters. It would be interesting to see whether there are differences in this respect between normal cells and tumor cells.

Modifications of the actinomycin chromophore have been achieved in different ways as is demonstrated by formulas **8–25**. Formula **8** represents an actinomycin. P symbolizes the peptide lactone rings of the actinomycin which served as starting material. Mild acid hydrolysis replaces the amino group of the chromophore of actinomycin with a hydroxyl group, giving a biologically inactive 2-desamino-2-hydroxy-actinomycin (formula **9**) (6). Thionyl chloride converts compound **9** into a crystalline, biologically inactive 2-desamino-2-chloro-actinomycin (compound **10**). Treatment of compound **10** with ammonia (7) regenerates the original actinomycin (formula **8**). Alternatively, if compound **10** is treated with a primary or secondary amine instead of ammonia, one obtains actinomycin derivatives **11** or **12** in which one or both hydrogen atoms of the amino group of the chro-



(8) **(9)** **(10)** **(11)** **(12)**

[2] Janda MG. Diplomarbeit, Universität Göttingen, 1961.

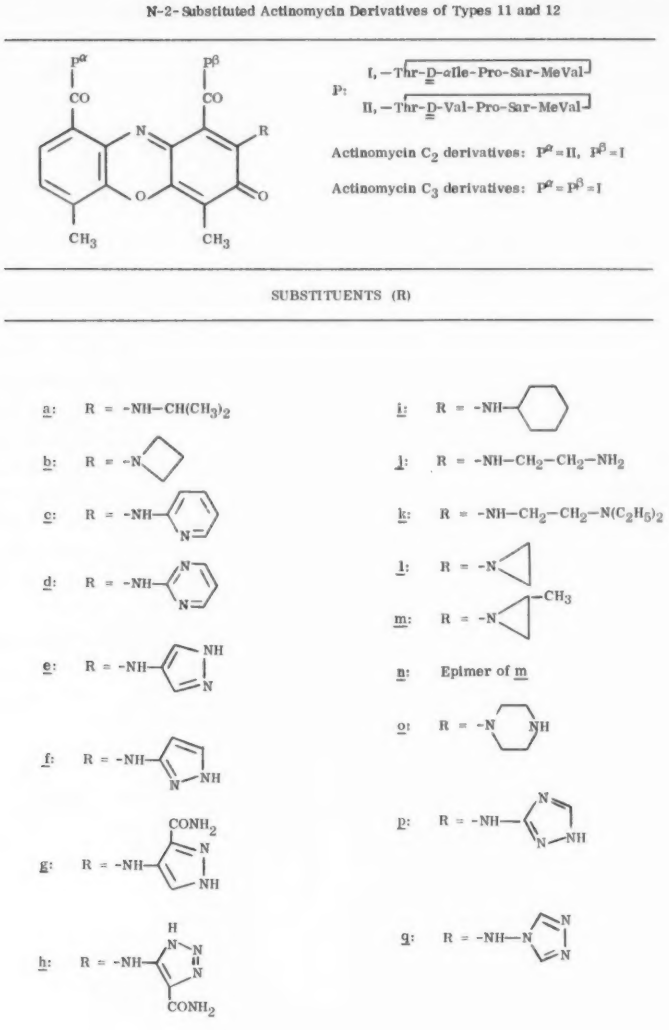
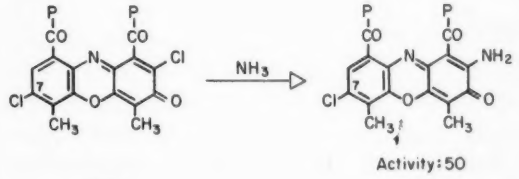

N-2-Substituted Actinomycin Derivatives of Types 11 and 12
P: I, —Thr-D-alle-Pro-Sar-MeVal—
II, —Thr-D-Val-Pro-Sar-MeVal—
Actinomycin C2 derivatives: Pα = II, Pβ = I
Actinomycin C3 derivatives: Pα = Pβ = I
SUBSTITUENTS (R)
a: R = -NH—CH(CH3)2
b: R = -N
c: R = -NH-
d: R = -NH-
e: R = -NH-
f: R = -NH-
g: R = -NH-
h: R = -NH-
i: R = -NH-
j: R = -NH—CH2—CH2—NH2
k: R = -NH—CH2—CH2—N(C2H5)2
l: R = -N
m: R = -N
n: Epimer of m
o: R = -N NH
p: R = -NH-
q: R = -NH-N
a-h: Actinomycin C2 derivatives
i-q: Actinomycin C3 derivatives

FIGURE 1.—N-2-substituted actinomycin derivatives of types 11 and 12. Adapted from Brockmann et al (9). Thr = threonine; Ile = isoleucine; MeVal = *N*-methylvaline; P = peptide. (*See* footnote 3 in the text.)

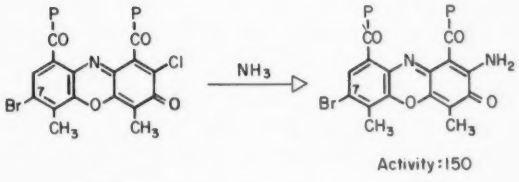
mophore are replaced by alkyl groups or other residues. In this way, we could prepare approximately 20 derivatives of actinomycins C_2 and C_3, respectively (8,9). The structures of 17 substituents that have been incorporated into N-2-substituted derivatives of types **11** and **12** are listed in figure 1. Most of them are inactive as antibiotics. Only the two actinomycin C_3 deriv-

atives **11k** and **11q** had about one tenth of the activity of actinomycin C_3. We suppose that a partial in vivo conversion into actinomycin is responsible for the biologic activity of these derivatives. In this connection, it should be mentioned that some of them (eg, the actinomycin C_2 derivative **12b**, fig 1) are partially transformed into the parent actinomycin on irradiation; this effect could be of therapeutic interest.

A second approach to actinomycins with a modified chromophore is represented by formulas **13–16**. If a 2-desamino-2-hydroxy-actinomycin (formula **9**) is treated with thionyl chloride in the presence of small amounts of water, a 2,7-dichloro-actinomycin (formula **13**) is obtained. Treatment of this compound with ammonia gives the 7-chloro-actinomycin (formula **14**) (10). In the case of actinomycin C_3, this 7-chloro derivative exhibits about one half of the antibiotic potency of the original actinomycin C_3. The corresponding 7-bromo-actinomycin is formed by treating a 2-chloro-actinomycin (formula **10**) with bromine. The resulting 2-chloro-7-bromo compound (formula **15**) gives, on treatment with ammonia, the corresponding crystalline 7-bromo-actinomycin (formula **16**) (10). In the case of actinomycin C_3, the antibiotic activity of this 7-bromo derivative is somewhat greater than that of the parent actinomycin C_3.

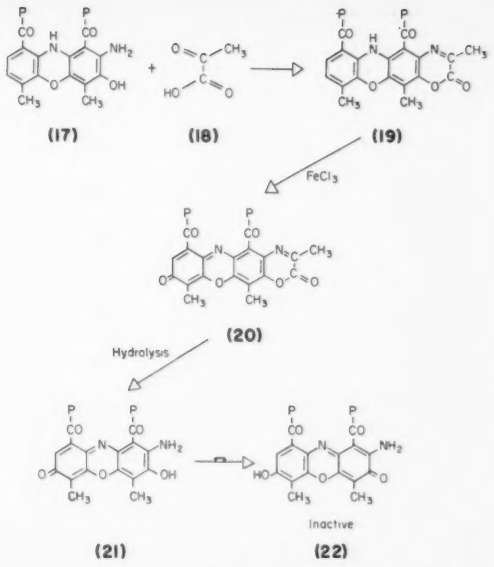


(13) (14)



(15) (16)

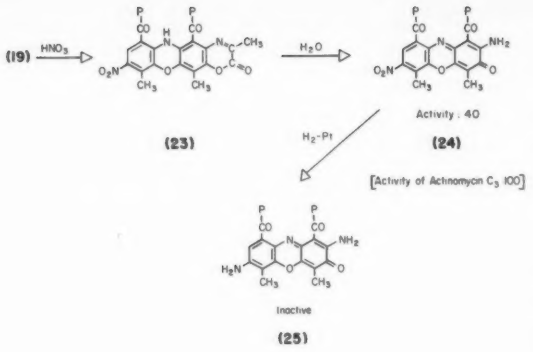
[Activity of Actinomycin C_3 : 100]



(17) (18) (19) (20) (21) (22)

A third way to prepare actinomycins substituted at carbon atom 7 of the chromophore is illustrated by formulas **17–22**. Hydrogenation transforms an actinomycin (formula **8**) into the corresponding dihydro-actinomycin (formula **17**), an ortho aminophenol, which is condensed with pyruvic acid (formula **18**) to give the oxazinone derivative **19**. In this way, the amino and oxo groups of the chromophore were protected so that we could oxidize the oxazinone (formula **19**) by means of ferric chloride to compound **20**. Compound **20** was hydrolyzed in the ferric chloride solution to give compound **21**, which was tautomerized to 7-hydroxy-actinomycin (formula **22**) (11). In the case of actinomycin C_3, this derivative was biologically inactive.

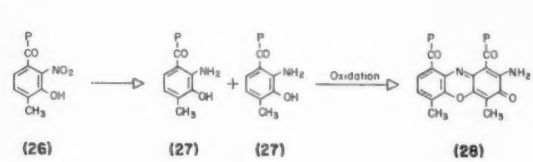
Starting with the oxazinone derivative **19**, we obtained also a nitration of the actinomycin chromophore. Treatment of compound **19** with dilute nitric acid gave the 7-nitro compound **23**. This compound was hydrolyzed in phosphate buffer (pH 7.2) to the crystalline 7-nitro-actinomycin (formula **24**) (11). 7-Nitro-actinomycin C_3 had nearly the same antibiotic activity as the parent material. Catalytic hydrogenation converted 7-nitro-actinomycin C_3 into crystallized 7-amino-actinomycin C_3 (formula **25**) (11), which had no antibacterial activity.



The aforementioned modifications of the actinomycin chromophore are limited by the fact that for the preparation of the derivatives all reactions must be carried out under conditions that do not harm the peptide rings or the chromophore group. From the very beginning, therefore, it has been obvious that these restrictions would be eliminated if actinomycins, as well as structural analogs, could be obtained by total synthesis. Our investigations have opened three routes for the synthesis of actinomycins and their analogs.

Actinocyl peptides such as compound 28 having the two carboxyl groups of the actinomycin chromophore actinocin (compound 2) bonded to the amino groups of two peptides were prepared in our laboratory (12) through the following steps:

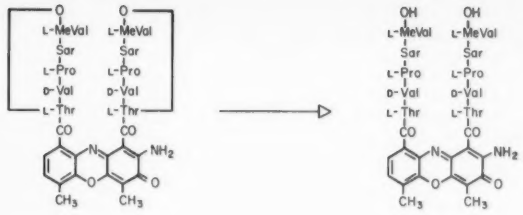
1. Synthesis of a peptide derivative in which the *N*-terminal amino acid of the peptide is acylated with 2-nitro-3-hydroxy-4-methylbenzoic acid to produce compound 26.
2. Reduction of compound 26 to provide the 2-amino-3-hydroxy-4-methylbenzoyl-peptide (compound 27).
3. Oxidative condensation of two molecules of compound 27 to give compound 28.



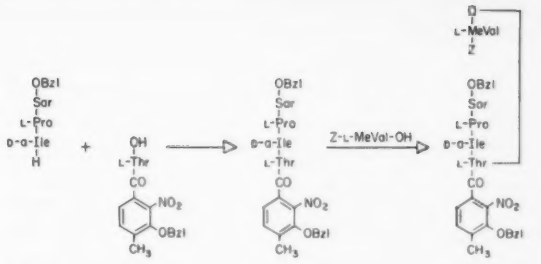
Applications of this procedure to actinomycin synthesis implied: (*a*) synthesis and oxidative condensation of an intermediate (compound 27) in which the peptide lactone ring of an actinomycin is *N*-acylated with 2-amino-3-hydroxy-4-methylbenzoic acid or (*b*) synthesis of an actinocinyl peptide (compound 28) in which the carboxyl groups of actinocin (compound 2) are bonded to an intermediate of an actinomycin peptide ring in such a manner that ring closure of the intermediate transforms the actinocinyl peptide into the desired actinomycin.

From the beginning of our work, it has been obvious that the critical key step of each actinomycin synthesis would be the closure of the peptide lactone ring. Concerning this reaction, the following questions had to be answered: (*a*) Should cyclization be attained by peptide bond formation or by lactonization? (*b*) At which stage of the synthesis should the rings be closed? Leads were obtained from two ring-opening reactions of the actinomycins.

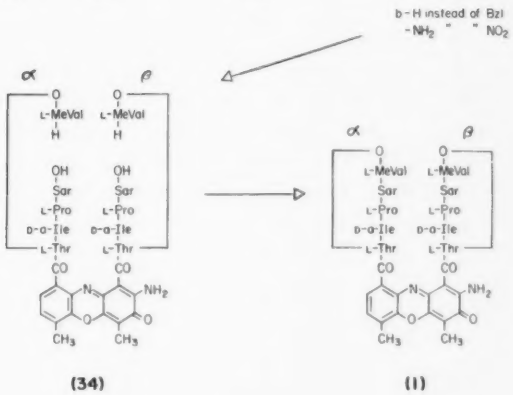
Treatment of an actinomycin (for example, actinomycin C_1 [formula 5] in its dihydro derivative form) with sodium methoxide at room temperature opens both peptide rings by hydrolysis of the two lactone groups to give the corresponding actinomycinic acid (formula 29), ie, an actinocinyl bis-pentapeptide. Our first plan, therefore, was to synthesize this actinomycinic acid (compound 29) and then close the two peptide rings by esterification of the terminal carboxyl groups with the hydroxyl groups of the threonine residues. Because this cyclization was expected to be the main difficulty in the synthesis, we decided to study this reaction first with actinomycinic acid prepared from actinomycin C_1 and not with the very precious synthetic material. However, experiments to prepare sufficient starting material for this purpose revealed that the opening of the peptide rings was accompanied by side reactions and that the resulting side products could not be separated satisfactorily.



(5) (29)



(30) (31) (32) (33a)



(34) (1)

While these experiments were under way, we found that treatment of actinomycin C_3 (formula **1**) with concentrated hydrochloric acid for a few hours at 40 C gave a degradation product of the structure **34** that was named bis-*seco*-actinomycin C_3 (13,14). Its formation indicated that concentrated hydrochloric acid attacks the peptide chains of actinomycin (preferentially at the bond between sarcosine and *N*-methylvaline). In contrast, dilute hydrochloric acid attacks preferentially the amino group of the chromophore giving 2-desamino-2-hydroxy-actinomycin (*see* conversion of compound **8** to compound **9**).

Each of the two peptide components of bis-*seco*-actinomycin C_3 (formula **34**) contains a carboxyl group (belonging to the sarcosine residue) and a methylamino group (belonging to the *N*-methylvaline residue). In other words, each peptide moiety possesses two groups capable of forming a peptide bond. Therefore, it was expected that reactions producing peptide bonds would cyclize each pentapeptide moiety through peptide bond formation and regenerate the actinomycin C_3 (formula **1**) from which the bis-*seco*-actinomycin C_3 (formula **34**) had been prepared. As we pointed out, this cyclization can be attained by means of dicyclohexylcarbodiimide (following the method of Sheehan[3]) or by means of ethyl chloroformate. We thus obtained crystallized actinomycin C_3 from bis-*seco*-actinomycin C_3 (15).

To develop this partial actinomycin synthesis into a total synthesis, we had to synthesize bis-*seco*-actinomycin C_3. This problem was solved in the following way (14,16,17). The tripeptide benzylester (formula **30**) was coupled with the L-threonine derivative (formula **31**) to give compound **32**. The next step was the esterification of the hydroxyl group of the threonine residue in compound **32** with *N*-benzyloxycarbonyl-L-*N*-methylvaline (Z–MeVal–OH).[4] This reaction could not be accomplished by conventional methods. The desired esterification was finally achieved by using the *N,N*-carbonyldiimidazole reagent first introduced for esterification by Staab.[5]

The compound **33a** thus obtained was hydrogenated in order to reduce the nitro group and to split off the two benzyl groups as well as the carbobenzoxy group. The resulting re-

[3] *See* Sheehan JC, and Hess GP. A new method of forming peptide bonds. *Journal of the American Chemical Society* 77:1067, 1955.

[4] Abbreviations for amino acids used in this paper conform to the recommendations of the International Union of Pure and Applied Chemistry-International Union of Biochemistry (IUPAC-IUB) Commission on Biochemical Nomenclature. (*See Journal of Biological Chemistry* 247:977–983, 1972.) Bzl = benzyl; P = peptide; Z = benzyloxycarboxyl.

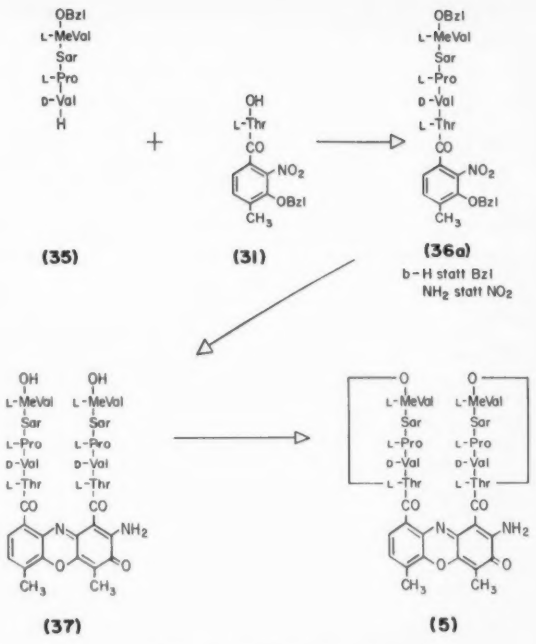
[5] *See* Staab HA. Neuer Weg zur Darstellung von Carbonsäure-estern. *Angewandte Chemie* 71:194, 1959.

duced product **33b** gave on oxidation by means of potassium ferricyanide a compound that proved to be identical to the bis-*seco*-actinomycin (formula **34**) prepared from actinomycin C_3 (formula **1**). By treating this synthetic bis-*seco*-actinomycin C_3 with ethyl chloroformate we obtained crystallized actinomycin C_3 (formula **1**) (14,16,17). Thus, we had attained the first total synthesis of an actinomycin.

In the meantime, our group had improved the hydrolytic conversion of actinomycin C_1 into actinomycinic acid (18). Because we were obtaining yields of approximately 80%, we had sufficient material for a detailed study of the recyclization of actinomycinic acid into actinomycin. In our efforts to attain a lactonization of actinomycinic acid into actinomycin, we had to keep in mind the following points: (*a*) the carboxyl groups had to be transformed into an active derivative, and (*b*) this transformation had to be attained in the presence of the two threonine hydroxyl groups that could be esterified. This is in contrast to a normal esterification in which the acid is first converted into an active derivative and then, in a second step, this derivative is treated with the alcoholic esterification partner.

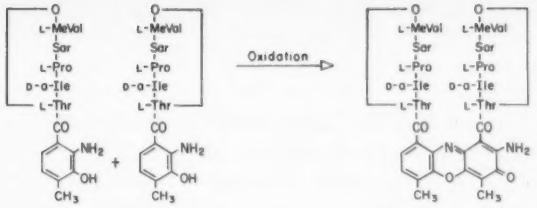
Furthermore, if cyclization was to succeed, three prerequisites had to be met: (*a*) a favorable conformation of the peptide chain in which the ring to be closed is preformed to the extent that the activated carboxyl group can contact the hydroxyl group of the threonine, (*b*) no steric interference with this contact by neighboring groups, and (*c*) no overriding competitive side reactions. From more than 200 experiments we learned that the cyclization of the C_1 actinomycinic acid (formula **37**) into actinomycin C_1 or D (formula **5**) could be attained only by means of acetylimidazole in acetyl chloride. Chromatography of the reaction product delivered a crystalline preparation that was identical to the starting actinomycin (formula **5**) used in preparing the actinomycinic acid. To develop this second partial synthesis of an actinomycin (18) into a total synthesis, we had to synthesize the actinomycinic acid (formula **37**). This was done in the following way.

Coupling of L-threonine with 2-nitro-3-benzyloxy-4-methylbenzoyl chloride gave the L-threonine derivative (formula **31**) that we had already used in the first actinomycin synthesis (14). This derivative was coupled with D-valyl-L-prolyl-sarcosyl-L-*N*-methylvaline benzyl ester (formula **35**). Catalytic hydrogenation of the resulting pentapeptide derivative **36a** and oxidative condensation of the reduction product **36b** by means of potassium ferricyanide delivered a preparation that was identical to the actinomycinic acid (formula **37**) prepared from actinomycin C_1 (formula **5**). Cyclization of this synthetic actinomycinic acid by treatment with acetylimidazole in acetyl chloride gave crystallized actinomycin C_1 (formula **5**) (19,20).



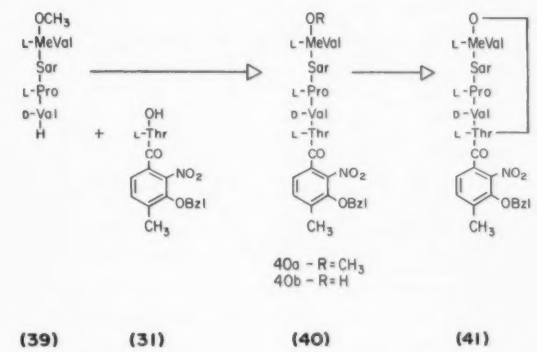
In 1956 we suggested in connection with our proposed structure **1** for actinomycin C_3 that the final step of the biosynthesis might be the oxidative condensation of two molecules of a precursor (formula **38**) to produce actinomycin C_3 (formula **1**) (21).

In this hypothetical route, cyclization of the two peptide moieties is not the last step as is the case in the syntheses via bis-*seco*-actinomycin (formula **34**) or actinomycinic acid (formula **37**). A synthesis using this pathway was developed in our laboratory for actino-

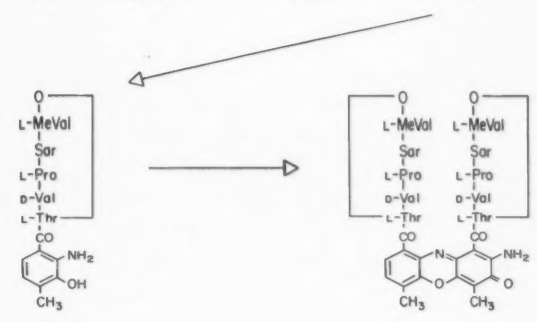


(38) (38) (1)

mycin C_1 (D) (formula 5) in the following manner (22,23). The first steps up to compound **40a** are the same as in the actinomycin synthesis via actinomycinic acid, except that in compound **40a** the residue *R* at the *N*-methylvaline residue is methyl instead of benzyl as in compound **36a**. Hydrolysis by means of dilute sodium hydroxide converted the methyl ester **40a** into the corresponding acid **40b**, which, on treatment with imidazole in acetyl chloride, gave the cyclic peptide derivative **41**. On hydrogenation, derivative **41** was converted into the precursor **42**, which, on mild oxidation by means



(39) (31) (40) (41)



(42) (5)

of potassium ferricyanide, gave crystallized actinomycin C_1 (D) (formula 5) (22,23).

On the basis of these results, we began with the synthesis of a number of actinomycin analogs via the corresponding actinomycinic acids. To decide in which respect the structure or configuration of these analogs should be different from those of the naturally occurring actinomycins, we had to consider several facts. The mechanism of the biologic action of the actinomycins had by that time been clarified by several groups (24–28). The actinomycins are antibiotic, toxic, and cancerostatic because they bind to the DNA double helix of the genes and, consequently, inhibit DNA-dependent RNA polymerase, thus suppressing the synthesis of messenger RNA. A prerequisite for this effect is an extremely slow dissociation of actinomycin from DNA or, in other words, a very long average lifetime of the complex between actinomycin and DNA.

On the basis of these facts, as well as the results of Hamilton, Fuller, and Reich (29), Cerami, Reich, Ward, and Goldberg (30), and their own hydrodynamic, kinetic, and equilibrium measurements, Müller (31) and Müller and Crothers (32) have shown that the formation, structure, and efficiency of the actinomycin-DNA complex can be described in terms of intercalation of the 2-aminophenoxazone-(3) ring system (ie, the actinomycin chromophore) between two base pairs (one of them guanosine-cytosine) of the DNA helix from the small groove so that the peptide rings project into the small groove where they assume positions of optimal interaction with the DNA backbone chain. In the most stable form of the complex, the peptide rings have undergone conformational changes that adapt their structure to interact specifically with the DNA backbone, each ring interacting with one strand of the double helix. The slow steps in the reaction of actinomycin with DNA are interpreted as arising from the mutual conformational adaptation of the oligopeptide rings and the DNA molecule, which is slow because the pentapeptide rings have such a tightly packed structure that conformational changes are sterically hindered.

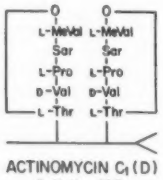
The Müller-Crothers model is able to explain differences in the biologic activity of the actino-

mycins and actinomycin derivatives. Our idea, therefore, was to synthesize a number of analogs that differ only slightly in structure or configuration from the natural actinomycins. It was of interest to explore how closely the model would agree with the biologic activity or inactivity of these new actinomycin analogs. Formulas 43–53 show the structures and configurations of our compounds and their activity against *Staphylococcus aureus* in the dilution test. In general, it can be said that this activity parallels the degrees of toxicity and anticancer effect.

In compound **43**, the L-threonine residue of actinomycin C_1 (D) (compound **5**) is replaced by L-serine (20,33). This replacement of the β-methyl group of threonine by hydrogen decreases the antibiotic activity from 100 to approximately 20. If the D-valine residue of actinomycin C_1 (D) is exchanged for D-alanine as in compound **44** (20) (ie, if the β-methyl groups of D-valine are replaced by hydrogen), the antibiotic activity decreases to about one hundredth of that for actinomycin C_1 (D).

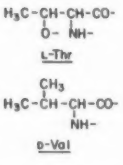
The actinomycin analog **45** (20) differs from actinomycin C_3 (formula **1**) only in the transposition of the β-methyl group of the D-*allo*-isoleucine residue of actinomycin C_3 (under loss of chirality) to the γ-carbon atom of the D-leucine residue in compound **45**. As a result, the activity falls to one fourth. Inversion of the configuration at all eight asymmetric carbon atoms of actinomycin C_1 (D) (formula **5**) as in the enantiomer **46** (34) leads to complete loss of antibiotic activity. Pseudo-actinomycin C_1 (D) (formula **47**), in which the peptide rings and the chromophore methyl groups of actinomycin C_1 (D) are mutually exchanged (35), was also totally inactive.

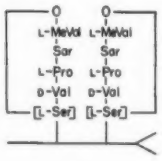
Starting with a mixture of α- and β-*seco*-actinomycin C_1 (D) in which only one peptide



ACTINOMYCIN C_1 (D)
Activity: 100

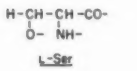
(5)

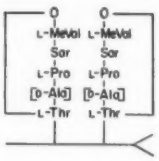




Activity: 20

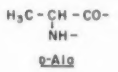
(43)

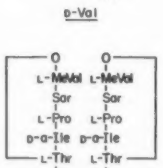




Activity: 1

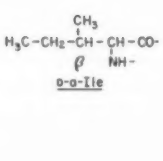
(44)

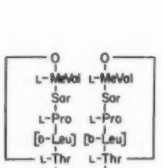




ACTINOMYCIN C_3
Activity: 100

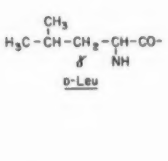
(1)

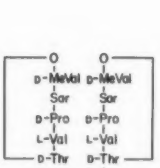




Activity: 25

(45)

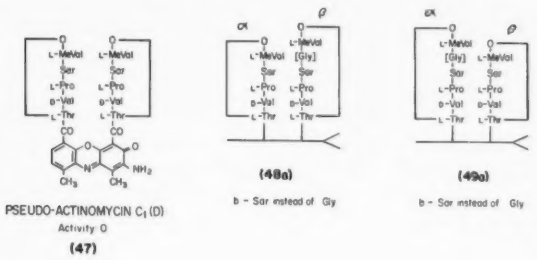


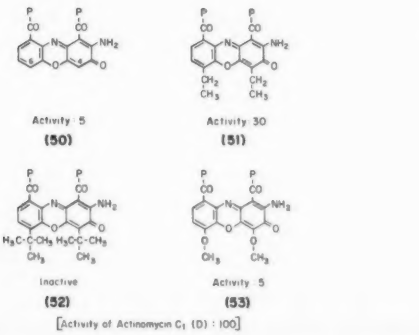


ACTINOMYCIN C_1(D)
ENANTIOMER
Activity: 0

(46)

ring is opened between the sarcosine and L-*N*-methylvaline residues of actinomycin C_1 (D) (formula 5) (13), we were able to enlarge one of the peptide rings by insertion of a glycine or sarcosine residue and to obtain in this way a mixture of the two isomers **48a** and **49a** as well as **48b** and **49b** (36). Both of these mixtures proved to be biologically inactive.





According to the intercalation model of the actinomycin-DNA complex, the insertion of the chromophore between the base pairs in the DNA structure should be sterically hindered by bulky substituents on the chromophore. Equilibrium measurements with the actinomycin derivatives **14**, **16**, and **24**, as well as **22** and **25** and derivatives of **22** and **25**, have revealed that small substituents in the 7-position (either electron attracting or electron donating) slightly increase the affinity of such actinomycin derivatives for DNA (31,32). Introduction of large substituents (by alkylation or acylation of the 7–OH and 7–NH_2 groups of compounds **22** and **24**, respectively) decreases the affinity. In this connection, it was of interest to synthesize the 4,6-position analogs **50**, **51**, **52**, and **53** of actinomycin C_1 (D) (37–39).

We found that substitution of hydrogen or methoxyl groups for the methyl groups in positions 4 and 6 (as in compounds **50** and **53**) decreases the antibiotic activity significantly, whereas two *tert*-butyl groups in place of the methyl groups of actinomycin C_1 (D) (compound 5) lead to complete loss of activity (as in compound **52**). If ethyl groups replace the methyl groups (as in compound **51**), the activity decreases to about one third. Consequently, methyl groups at carbons 4 and 6 of the chromophore are optimal for the biologic activity.

In conclusion, it can be said that all observations concerning the antibiotic activity of our actinomycin derivatives and actinomycin analogs and all measurements of Müller and Crothers are in agreement with the actinomycin-DNA intercalation model proposed by these authors (32).

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Synthesis of Peptide Analogs of Actinomycin D (NSC-3053) [1,2,3]

Johannes Meienhofer [4,5]

SUMMARY

The rationale of searching for actinomycins with improved cancer chemotherapeutic properties through chemical synthesis of peptide analogs has been based on the importance of the pentapeptide lactone moieties for (*a*) the unique solubility properties of actinomycins, (*b*) the biologic potencies of actinomycins, and (*c*) the complex formation with double-stranded DNA in cell nuclei. In designing new peptide analogs, consideration should be given to making minimal changes and preparing either "isosteric functional analogs" or "isofunctional steric analogs," and to replacing amino acid residues in positions 1′ and 4′ which remain unchanged among various naturally occurring and biosynthetic actinomycins. Total synthesis of actinomycin D has been carried out with the aim of developing an effective synthetic pathway, which is characterized by performing cyclization of the pentapeptide lactones by peptide bond formation between proline and sarcosine. Pathways for the preparation of useful protected derivatives of L-α,β-diaminopropionic acid and of L-*threo*-α,β-diaminobutyric acid for syntheses of lactam analogs have been developed. Syntheses of [di-(1′-L-α,β-diaminopropionic acid)]-actinomycin D (compound 6, *see* fig 8) and of [di-(1′-L-*threo*-α,β-diaminobutyric acid)]-actinomycin D (compound 7) followed the synthetic pathway established for actinomycin D. Cyclization failed in an attempted synthesis of [di-(4′-L-*N*-methylalanine)]-actinomycin D, possibly because of steric interference by the β-methyl group. Actinomycin D lactam (compound 7) effected only transient suppression of tumor growth in Ridgway osteogenic

[1] Part X of the series Synthesis of Actinomycin and Analogs. For Part IX, *see* ref 2.

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[3] Abbreviations used in this paper follow the rules of the IUPAC-IUB Commission on Biochemical Nomenclature. *See Biochemistry*, vol 5 (pp 1445–1453, 2485–2489, 1966) and vol 6 (pp 362–364, 1967); and the *Journal of Biological Chemistry*, vol 247 (pp 977–983, 1972):

Ac = acetyl; $\overset{Cl}{\underset{|}{A}}$la = L-β-chloroalanine; Boc = *tert*-butyloxycarbonyl; Bu^t = *tert*-butyl (ether); Bzl = benzyl (ether); Dbu = L-*threo*-α,β-diaminobutyric acid; Dpr = L-α,β-diaminopropionic acid; MA = mixed anhydride; MeVal = L-*N*-methylvaline; OBu^t = *tert*-butyl ester; OMe = methyl ester; ONp = *p*-nitrophenyl ester; ONSu = *N*-hydroxysuccinimide ester; OS(ONp)$_2$ = di-(*p*-nitrophenyl) sulfite; ROS = Ridgway osteogenic sarcoma; TFA = trifluoroacetic acid; Tos = *p*-toluenesulfonyl; and Z = benzyloxycarbonyl.

[4] Children's Cancer Research Foundation, and Department of Biological Chemistry, Harvard Medical School, Boston, Mass.

[5] I thank my collaborators Annemarie Fabis-Krischer, Y. Sano, Andrea Seki-Viano, Edith Judkins, R. Cotton, A. Trzeciak, R. P. Patel, C. G. Glaser, E. Atherton, K. Kuromizu, S. Moore, M. Kondo, and Michelle Copeland for their contributions, and Dr. G. E. Foley and Barbara L. Brown for the in vitro and in vivo biologic assay data respectively.

sarcoma in mice during administration of the drug at 10–20-fold higher doses than actinomycin D. The lower lactam homolog (compound 6) was about one third to one half as active as actinomycin D and warrants further investigation.

[Cancer Chemother Rep 58:21–34, 1974]

In 1965, Dr. Sidney Farber encouraged us to develop a program of chemical modification and synthesis of actinomycin analogs with the aim of obtaining antitumor agents which might possess improved chemotherapeutic properties as compared with actinomycin D.[*] Improvements were not only sought in terms of higher therapeutic indices, but also with respect to exerting clinically useful therapeutic effects against those human tumors (preferentially the more commonly occurring ones) that are not attacked by the actinomycins which are available from natural sources (1). Since then, we have been engaged in the preparation of peptide analogs of actinomycin D (2–8). This has also been a major concern in the laboratory of Professor H. Brockmann since 1960 (9–17), and he has reviewed his progress superbly at this Conference (18).

RATIONALE FOR SYNTHESIZING ACTINOMYCIN PEPTIDE ANALOGS

The rationale for synthesizing peptide analogs in the search for actinomycins with improved altered therapeutic characteristics is based on observations (*a*) that the peptide moieties are responsible for the unique solubility properties of actinomycins, (*b*) that rather large potency differences exist between naturally occurring actinomycin peptide analogs, and (*c*) that the pentapeptide lactone rings play important, yet unknown, roles in the interaction of actinomycin with double-helical DNA (19–21).

The unusual solubility behavior of actinomycin was first studied by Berg et al (22) and subsequently by Schulmeyer (23), Müller and Emme (24), and Crothers et al (25). The large negative temperature coefficient of actinomycin D solubility in water (fig 1) indicates a strong hydrophobic nature of the pentapeptide lactones. We have determined the water solubility of actinomycin D to be 0.8 mg/ml at 20 C and 128 mg/ml at 1 C. An even larger increase in solubility with decreasing water temperature was observed for actinomycin C_3 with a solubility of 200 mg/ml at 1 C (23). In most organic solvents, actinomycin D is readily soluble, which shows positive temperature dependence (fig 1). These solubility properties are solely attributable to the peptide lactone rings; the chromophore itself, 2-amino-4,6-dimethylphenoxa-

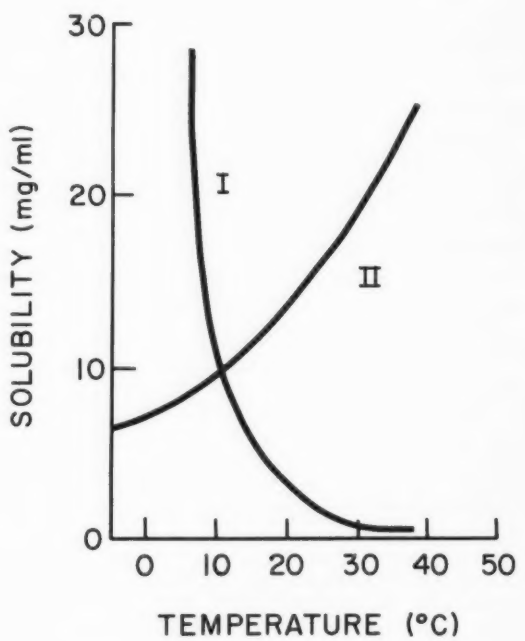
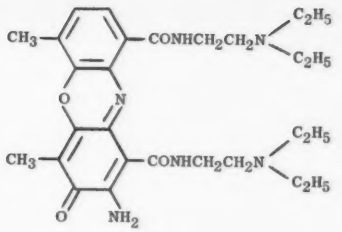


FIGURE 1.—Temperature dependence of solubility of actinomycin D. I = Solubility in water; II = solubility in methanol. From Berg et al (22).

[*] For the structure of actinomycin D *see* figure 1 in the Preface to this series of papers (Cancer Chemother Rep 58:1–3, 1974) and figure 3 of this paper.

zine(3)one-1,9-dicarboxylic acid, is virtually insoluble in most solvents. Solubility of actinomycins in both hydrophilic and lipophilic media should be significant for intracellular and intercellular transport phenomena in organisms, including cell membrane permeation during uptake of the drug. It can be expected that peptide analogs possess variously modified solubility properties.

The importance of the pentapeptide lactones for the biologic activity of actinomycin is also apparent from the rather large differences in potency among the naturally occurring variants of known structures, all of which contain the same chromophore. Table 1 shows data from studies by Reich et al (26) and by Müller and Crothers (20) comparing inhibition of HeLa cell division, bacteriostatic activity against *Bacillus subtilis*, and DNA binding constants, as determined by equilibrium dialysis, for several naturally occurring actinomycins. Specific activities differ by factors of 15–50 in inhibition of *B. subtilis* and HeLa cell growth and by a factor of about 10 in DNA binding constants. Significantly, actinomycin X_2 exhibits two to three times higher inhibitory potencies than actinomycin D. These actinomycins differ in the 3 position of the β peptide lactone: actinomycin X_2 possesses a 4-oxoproline residue and actinomycin D possesses a proline residue.



COMPOUND 1.—Actinomine.

The important role of the two cyclic pentapeptide lactones in complex formation between actinomycins and DNA is apparent from the large differences, amounting to two to four orders of magnitude, in the rates of complex formation and dissociation (20) between actinomycin D and related simple chromophore derivatives such as actinomine (compound 1), the diethylaminoethylamide of actinocine, ie, 2-amino-4,6-dimethylphenoxazine(3)one-1,9-di-(diethylaminoethyl carboxamide). Smaller, but significant differences have been observed between the natural actinomycin variants, C_1, C_3, and X_2; synthetic peptide analogs with altered DNA-complexing properties might, therefore, be of therapeutic interest. During complex formation the peptide rings appear to undergo

TABLE 1.—Comparison of cytotoxicity, bacteriostatic potency, and DNA binding capacity of several naturally occurring actinomycins*

Actinomycin	Inhibition of HeLa cell division, MID† (2.5×10^4 cells/dish; μg/ml)	Inhibition of *B. subtilis* growth, ID100 (μg/ml)	Equilibrium constants of binding to calf thymus DNA (K_{sp} [M^{-1}] at 20 C)‡
X_2	0.01	0.1	1.1×10^7
C_1(D)	0.02	0.25	2.3×10^6
C_2	0.02	0.25	4.9×10^6
C_3	0.02	0.25	2.4×10^6
X_{os}	0.02	0.35	3.8×10^6
$X_{o\beta}$	0.50	1.50	1.3×10^6

*Data selected from Reich et al (26) and Müller and Crothers (20).

†MID = median inhibitory dose.

‡In buffer containing 0.08 M Na_2HPO_4, 0.02 M NaH_2PO_4, 0.18 M NaCl, and 0.01 M di-Na EDTA (pH 6.9).

conformational changes which adapt their structures to interact optimally with the DNA double helix, and one of the functions of the cyclic pentapeptides is to provide a means for obtaining a slowly dissociating complex (20).

Effects of chemical modifications at various positions of the actinomycin molecule on its bacteriostatic potency have been extensively investigated in the laboratory of Brockmann (18). More than 60 derivatives have been prepared by chromophore modification at the 2-amino group and the 7-carbon atom and by *O*-acylation of the peptides of hydroxyproline-containing actinomycins (for a review, *see* ref 1). With a few exceptions, these changes in the native molecule have resulted in reduced bacteriostatic potency, as schematically shown in figure 2. From a perusal of the accumulated information it seemed that preparation of peptide analogs by total synthesis should be a more promising approach toward development of actinomycins with improved chemotherapeutic properties.

CONSIDERATIONS IN DESIGNING PEPTIDE ANALOGS

Considerable experience has been accumulated in designing analogs of biologically active peptides (27,28). Interpretation of differences in the biologic properties of analogs is facilitated if minimal structural changes are made (29), ie, with isosteric functional analogs (in which chemically reactive groups have been substituted) and with isofunctional steric analogs (in which chemically inert space-filling groups have been replaced).

All natural and biosynthetic actinomycins possess two common amino acids: the threonine residues [7] in the 1′ position and the sarcosine residues in the 4′ position. We are interested in preparing analogs by chemical synthesis with amino acid substitutions in either of these positions which do not seem to experience variations during biosynthesis of actinomycins. It is also deemed to be desirable to chemically incorporate such residues that are foreign to the biosynthetic process. An outstanding example has been the synthesis by du Vigneaud et al (31–34) of deamino-oxytocin, an analog which possessed almost double the oxytocic potency of oxytocin. Biosynthesis of peptide antibiotics such as gramicidin and tyrocidin proceeds via nonribosomal pathways by multienzyme complexes through pantothenate-assisted sequences of thioester-activated peptide condensations (35). The enzymes involved in the biosynthesis of actinomycin peptide lactones (36–38) have yet to be identified; however, sufficient information has been gathered on their substrate specificity which will allow us to choose such amino acid residues for chemical analog synthesis that cannot be utilized by the enzymes during biosynthesis.

From these considerations we planned to investigate the role of the lactone ring oxygens through the preparation of isosteric lactam and thiolactone analogs of actinomycin D in which the oxygen would be replaced by NH and by sulfur (fig 3). We hoped this might result in analogs with increased hydrophilicity or increased lipophilicity respectively.

DEVELOPMENT OF A USEFUL SYNTHETIC PATHWAY

Total synthesis of actinomycin peptide analogs requires approximately 20 consecutive chemical reactions starting from individual amino acids. Biologic activity can be expected only after completion of the entire synthesis. Absence of activity then might be due either to an inherent property of a new analog or to defects in synthetic procedures. Therefore, it was advisable to develop first an efficient synthetic pathway through synthesis of the parent actinomycin D. Its known physical and biologic properties permitted evaluation of each synthetic avenue. We have investigated eight different approaches including the approach of Brockmann and Lackner (12) via intermediate actinomycinic acid (compound 2). During these studies several derivatives of actinomycinic acid were prepared (fig 4) in efforts to determine whether the known lack of biologic activity of the acid was due to the ionizable carboxyl groups and/or to the hydroxyl groups of threonine or to the loss of essential conformation. Both actinomycinic acid D di-(*tert*-butyl ester)

[7] Some actinomycins of the Z group appear to be exceptional in possessing one *N*-methylthreonine residue (1,30).

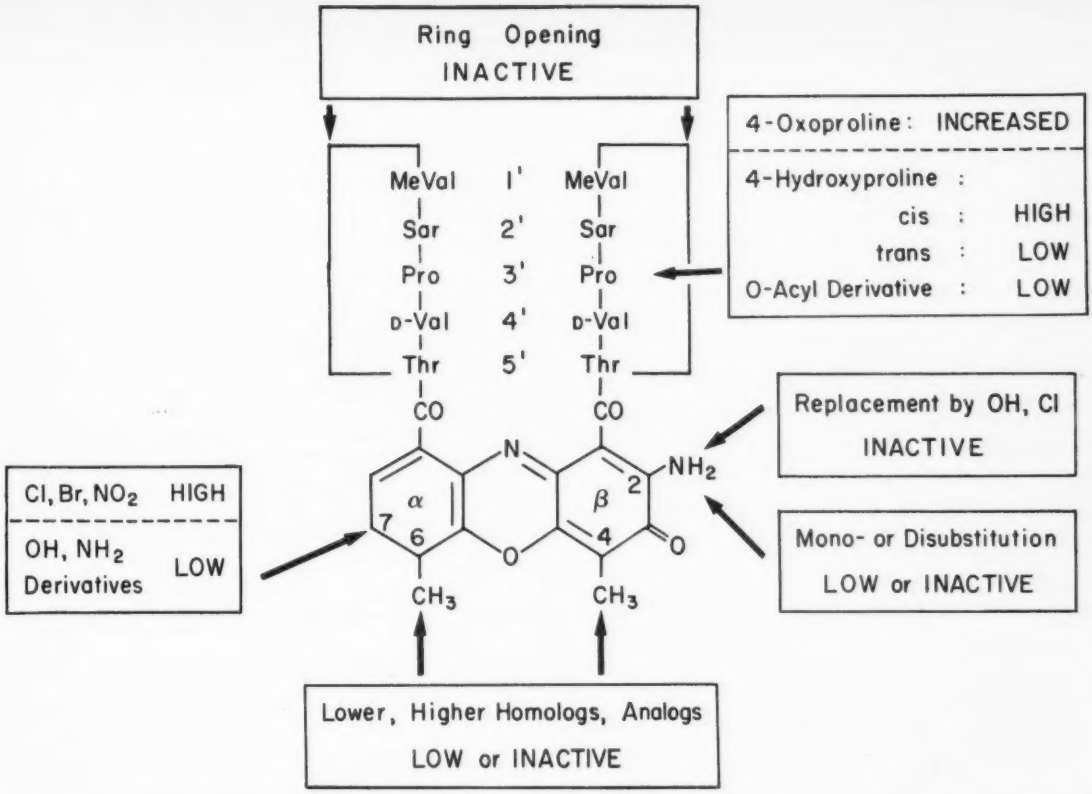


FIGURE 2.—Schematic survey of effects of modifications at different positions of the molecule on the bacteriostatic potency of actinomycin.

(compound 3, *see* fig 4) and the fully protected di-*O*-*tert*-butyl)-actinomycinic acid D di-(*tert*-butyl ester) (compound 4, *see* fig 4) were found to be devoid of antibacterial activity against *Lactobacillus arabinosus* and *L. fermenti*, an additional indication that it is the conformation of the cyclic lactone structures which is essential for the biologic activity of the actinomycins.

The pathway for synthesis of actinomycin D which produced the best results in our laboratory (6,7,40) is characterized by attaining the key reaction, ie, cyclization of the pentapeptide lactones, by peptide bond formation between proline and sarcosine using *p*-nitrophenyl ester activation (41,42), as depicted in figure 5. Crystalline actinomycin D was almost quantitatively obtained from the intermediate crystalline (2-nitro-3-hydroxy-4-methylbenzoyl)-L-threonyl-D-valyl-L-prolyl-sarcosyl-L-*N*-methyl-valine(threonine hydroxyl) lactone by catalytic hydrogenation and oxidative phenoxazinone formation. Recrystallization from ethyl acetate-hexane gave red trigonal prisms, possessing physical characteristics which compared favorably with natural actinomycin D.[8] Microbiological assays and toxicologic and antitumor assays in mice all showed the synthetic product to be indistinguishable from natural actinomycin D. In this manner, approximately 1 g of synthetic actinomycin D was produced. In this synthesis frequent use was made of column chromatography on Sephadex LH-20 in ethanol (or methanol) to separate complex reaction mixtures of protected peptides and to remove undesired

[8] Actinomycin D (lots 3008A-30B and 1687C-41AII) from Lederle Laboratories Division, American Cyanamid Co., Pearl River, NY.

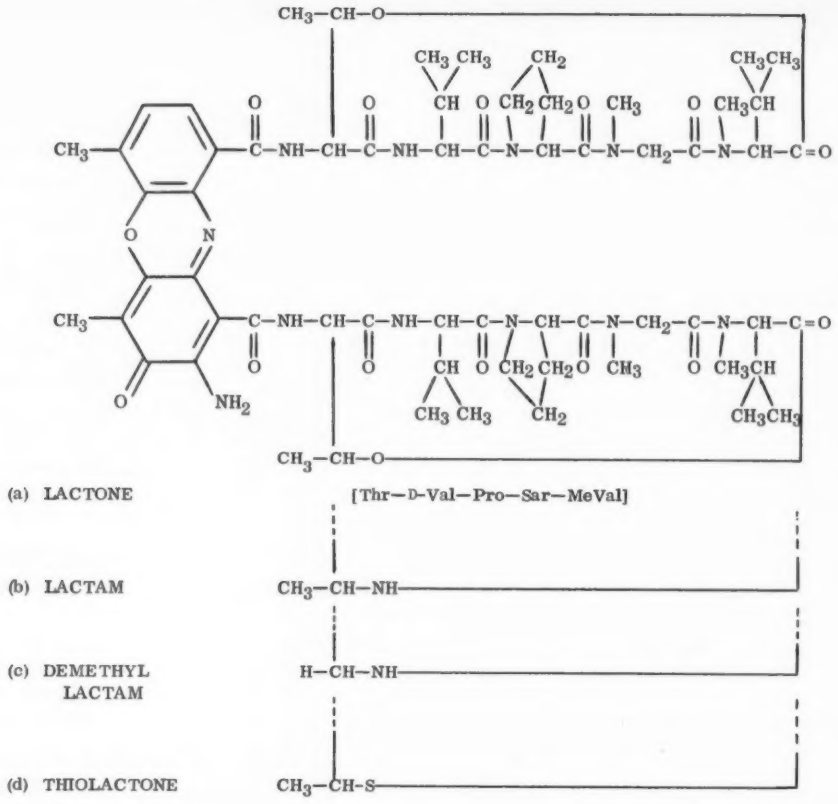
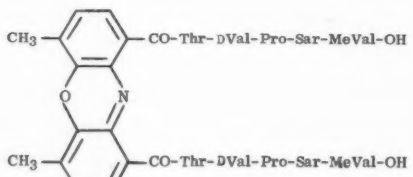


FIGURE 3.—Structures of actinomycin D (C_1) [a] and of lactam [b,c] and thiolactone [d] analogs.

side products (4). This technique which fractionates by molecular size allowed facile purification of gram amounts of protected peptides in single runs.



COMPOUND 2.—Actinomycinic acid D (C_1).

SYNTHESIS OF ACTINOMYCIN LACTAM ANALOGS

After the development of an effective synthetic pathway, synthesis of actinomycin peptide analogs could be tackled. For synthesis of isosteric actinomycin D lactam (fig 3b, fig 6, R = CH_3), the threonine residues had to be replaced by residues of L-*threo*-α,β-diaminobutyric acid, the synthesis of which proved to be difficult and time consuming. For this reason we decided to prepare first the lower homolog or di-desmethyl lactam (fig 3c, fig 6, R = H) in which threonine would be replaced by the simpler L-α,β-diaminopropionic acid which possesses only one symmetric carbon atom. The most suitable starting derivative for synthesis

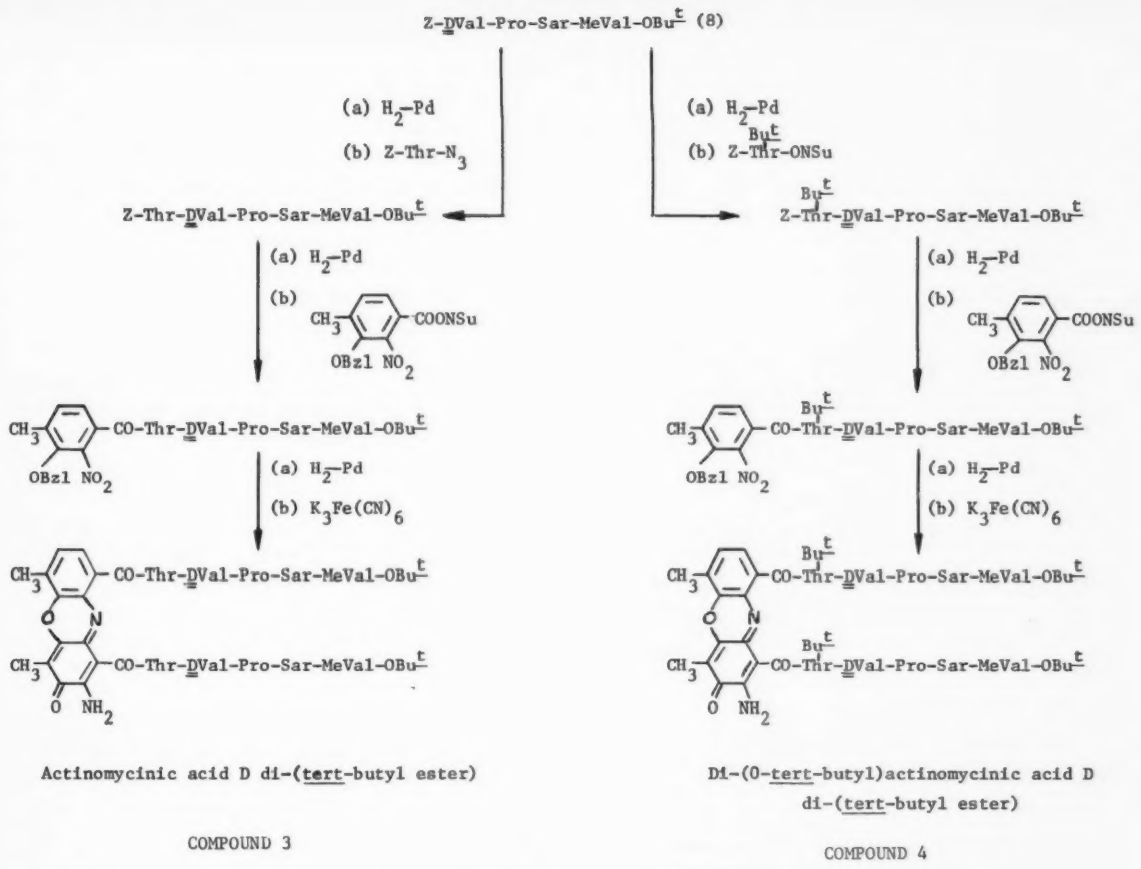


FIGURE 4.—Synthesis of actinomycinic acid D (8), its di-*tert*-butyl ester (compound 3), and di-(*O*-*tert*-butyl)-actinomycinic acid D di-(*tert*-butyl ester) (compound 4) (ref 6).

of the di-desmethyl lactam analog (compound 6, *see* fig 8) was found to be $N\alpha$-benzyloxycarbonyl-L-α,β-diaminopropionic acid methyl ester hydrochloride (compound 5, *see* fig 7). This was prepared either from L-serine methyl ester via benzyloxycarbonyl-L-β-chloroalanine (fig 7*a*) (5), or, preferably, from $N\alpha$-tosyl-L-asparagine via intermediate protection of the β-amine function by the *tert*-butyloxycarbonyl group (fig 7*b*).[9]

The analog preparation followed the pathway shown in figure 8, which was similar to that elaborated for actinomycin D (fig 5) and was characterized by peptide cyclization between proline and sarcosine. The cyclization reaction yielded, in addition to the desired compound (compound 8), a closely related side product,[10] which was difficult to separate (requiring repeated Sephadex LH-20-ethanol column chromatography) and which became the sole product

[9] Meienhofer J, Moore S, Patel RP, et al. Synthesis of [di-(1′-L-*threo*-diaminobutyric acid)]-actinomycin D. Paper in preparation.

[10] Compound 8 had an Rf value of 0.55 on tlc (silica gel; *sec*-butanol:formic acid:water, 150:27:23); the side product had an Rf value of 0.65.

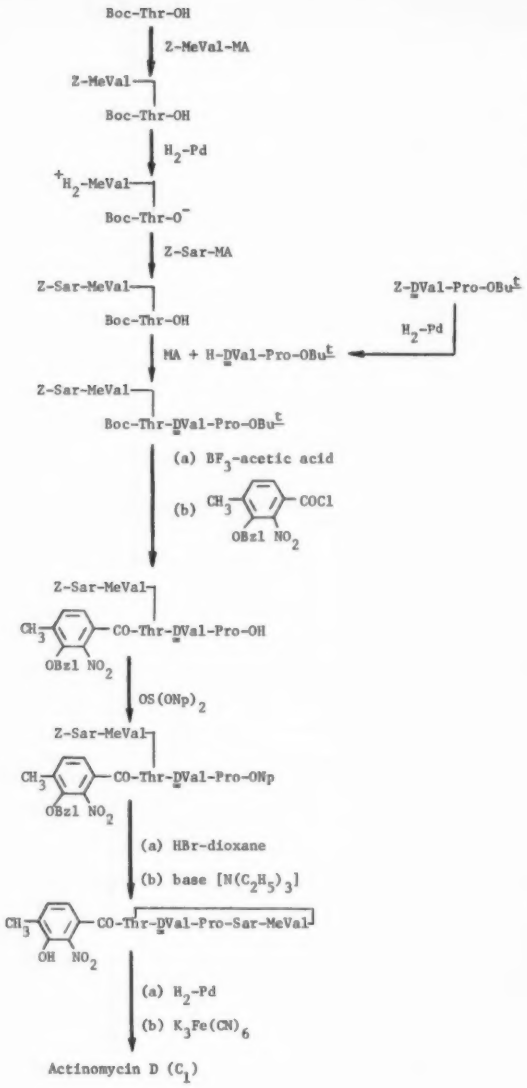


FIGURE 5.—Synthesis of actinomycin D (C_1) via peptide cyclization between proline and sarcosine. OS-(ONp)$_2$ = di-(*p*-nitrophenyl) sulfite.

at reaction temperatures above 55 C. Cyclization had to be performed at temperatures between 45 and 50 C to obtain compound 8 as the main cyclic product. The 2-nitro-3-benzyloxy-4-methylbenzoyl group was best introduced via the symmetrical anhydride (4), and the ensuing *N*α-(2-nitro-3-benzyloxy-4-methylbenzoyl)-L-α,β-diaminopropionyl-D-valyl-L-prolyl-sarcosyl-L-*N*-methylvaline(*N*β-diaminopropionic acid) lactam was obtained in crystalline form. Catalytic hydrogenation followed by oxidative phenoxazinone formation afforded the 1′,1′-di(ω-desmethyl) lactam analog (compound 6) as an orange-red powder (5).[9] The amount produced (10 mg) permitted preliminary biologic testing of this analog. It possessed approximately 10% of the bacteriostatic potency of actinomycin D, while its antitumor potency against Ridgway osteogenic sarcoma in mice ranged from 20% to 40% (table 2). Preparation of more material for detailed evaluation is presently in progress.

Considerably higher antitumor activity than that of the di-desmethyl lactam analog (compound 6) was expected from the isosteric lactam analog (compound 7, *see* fig 6, R = CH_3), by analogy with the large potency difference between the less active di-(1′-L-serine) analog and actinomycin D (C_1) (10,12). For synthesis of compound 7, suitable derivatives of L-*threo*-α,β-diaminobutyric acid were required. We found (43,44), in the meantime, that these were obtainable from L-threonine by a sequence of reactions shown in figure 9. Successive treatments of *N*,*O*-ditosyl-L-threonine methyl ester with ammonia-saturated methanol and 6-N hydrogen chloride gave, after several recrystallizations from water, *N*α-tosylα,β-diaminobutyric of the same configuration as the starting threonine. Presumably, the sulfonamide moiety influenced the steric course of the reaction causing a double inversion of the Cβ asymmetry center via intermediate aziridine formation.

*N*α-Benzyloxycarbonyl-L-*threo*-α,β-diaminobutyric acid methyl ester hydrochloride (compound 9, *see* fig 9) was the starting compound for the synthesis (2) of actinomycin D lactam (compound 7), which followed exactly the synthetic pathway (fig 8) used for the preparation of the lower homolog (compound 6). Cyclization to give

Z-Dbu-DVal-Pro-Sar-MeVal

proceeded without complications in close to 40% yield. Subsequent attachment of the chromophore precursor produced crystalline *N*α-

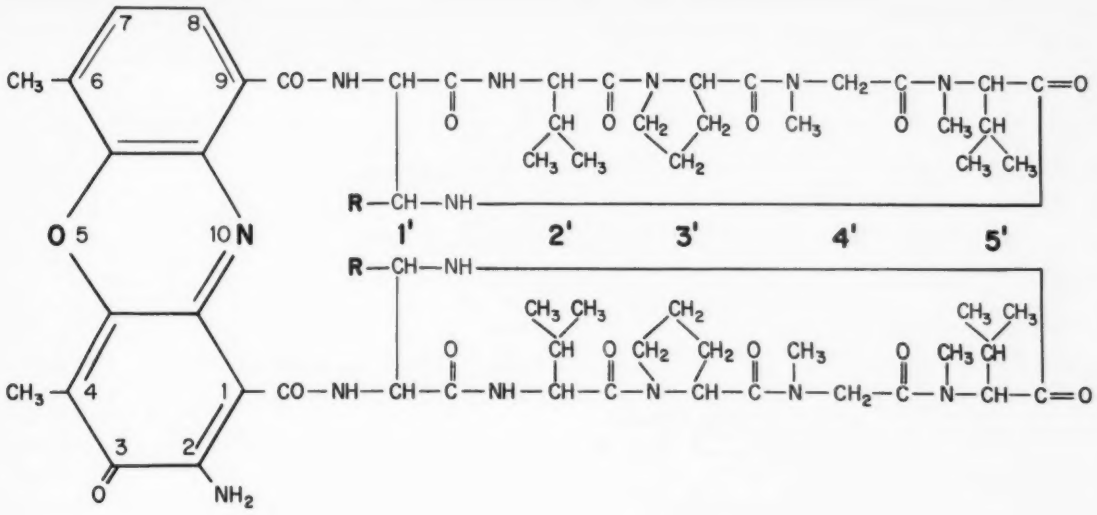


FIGURE 6.—Structure of actinomycin D lactam analogs. R = H: [di-(1'-L-α,β-diaminopropionic acid)]-actinomycin D (compound 6); synonym: di-(1'-ω-desmethyl)-lactam analog of actinomycin D. R = CH_3: [di-(1'-L-threo-α,β-diaminobutyric acid)]-actinomycin D (compound 7); synonym: actinomycin D lactam.

(2-nitro-3-benzyloxy-4-methylbenzoyl)-L-*threo*-α,β-diaminobutyryl-D-valyl-L-prolyl-sarcosyl-L-*N*-methylvalyl(*N*β-diaminobutyric acid) lactam in high yield. However, the chromophore formation through ferricyanide oxidation (45) of intermediate 3-hydroxy-4-methylanthraniloyl pentapeptide lactam did not proceed well in repeated experiments and yields ranged between 15% and 20% in contrast to quantitative reaction of the corresponding lactone to give actinomycin D (7). The bacteriostatic potency of actinomycin D lactam (compound 7) (table 2) was not higher than that of the di-desmethyl lactam analog (compound 6), and the antitumor

TABLE 2.—Biological activity and solubility characteristics of lactam analogs of actinomycin D*

Compound	Hydrophilicity in tlc, silica gel *s*-BuOH-HCOOH-H_2O (150:27:23)	Antibacterial potency: *L. arabinosus* and *L. fermenti*, ID50 (μg/ml)	Antitumor activity: ROS in AKD_2F_1 mice, MED (μg/kg/day × 4, ip)
Actinomycin D	Standard	0.03	60
Demethyl lactam (compound 6)	More hydrophilic	0.3	150–300
Lactam (compound 7)	More hydrophilic	0.4	600–1200 (temporary suppression during administration [LD100 at 2000])

*s-BuOH = *sec*-butanol; ROS = Ridgway osteogenic sarcoma; tlc = thin-layer chromatography; MED = minimum effective dose.

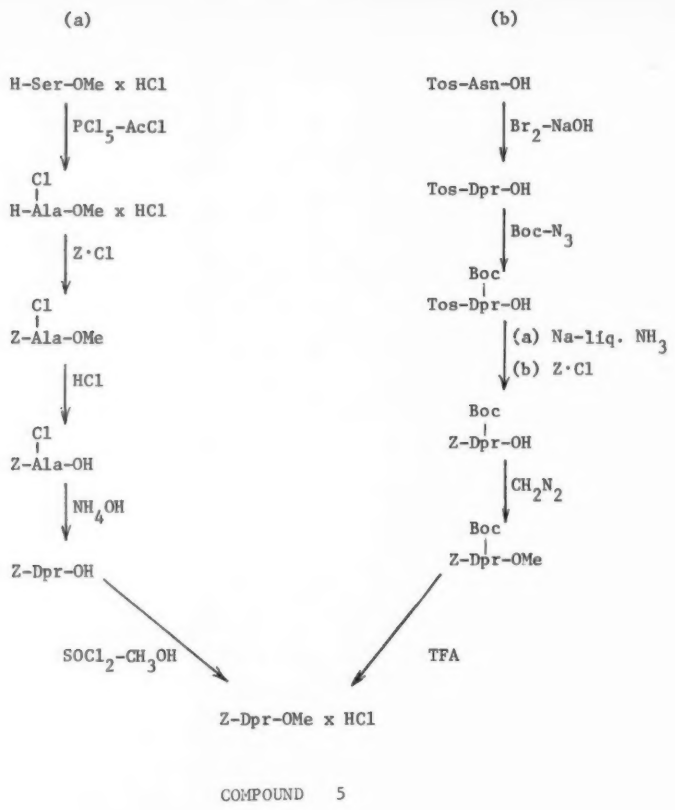


FIGURE 7.—Synthetic pathways for preparation of $N\alpha$-benzyloxycarbonyl-L-α,β-diaminopropionic acid methyl ester hydrochloride (compound 5); (*a*) starting from L-serine methyl ester, (*b*) starting from *p*-toluenesulfonyl-L-asparagine. Ala(Cl) = L-β-chloroalanine; Dpr = L-α,β-diaminopropionic acid.

activity was considerably lower and transient. These low activities rendered actinomycin D lactam uninteresting for further study. It is worth noting that structure-activity correlations between actinomycin lactone analogs do not seem to relate to those between corresponding lactam analogs.

SYNTHESIS OF 4′-POSITION PEPTIDE ANALOGS

The sarcosine residues in the 4′ positions are common to all naturally occurring and biosynthetic actinomycins of known structure (1). We were interested in studying the effects of replacing the 4-sarcosine residues of actinomycin D by homologous *N*-methylamino acids on the biologic activity of the drug. Work on a synthesis of [di-(4′-L-*N*-methylalanine)]-actinomycin D has been carried out [11] following the general synthetic route developed for the preparation of actinomycin D (fig 5). Z-MeAla-OH was coupled to the didepsipeptide Boc-Thr (MeVal)-OH. The ensuing tridepsipeptide, *O*-(benzyloxycarbonyl-L-*N*-methylalanyl-L-*N*-methylvalyl)-*N*-*tert*-butyloxycarbonyl-L-threonine (compound 10), was condensed with H-D Val-Pro-OBu^t to produce *O*-(benzyloxycar-

[11] Atherton E, and Kuromizu K. Unpublished data.

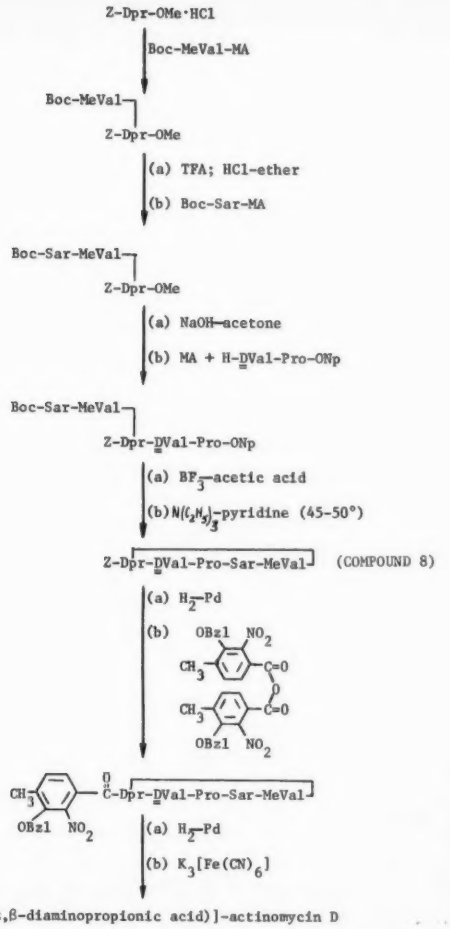
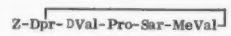


FIGURE 8.—Synthetic pathway used for preparations of lactam analogs of actinomycin D (refs 2,3,5; footnote 9).

bonyl-L-*N*-methylalanyl-L-*N*-methylvalyl)-*N*-*tert*-butyl-oxycarbonyl-L-threonyl-D-valyl-L-proline *tert*-butyl ester (compound 11). Removal of the *tert*-butyl protecting groups by boron trifluoride in acetic acid and condensation with 2-nitro-3-benzyloxy-*p*-toluic anhydride produced



COMPOUND 8

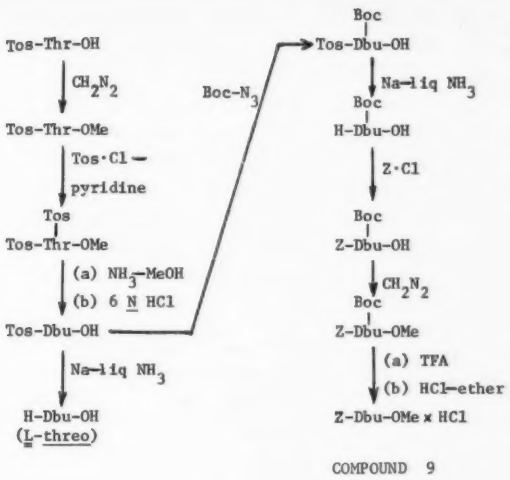


FIGURE 9.—Preparation (43,44) of L-*threo*-α,β-diaminobutyric acid (Dbu) from L-threonine and of *N*α-benzyloxycarbonyl-L-*threo*-α,β-diaminobutyric acid methyl ester (compound 9).

O-(benzyloxycarbonyl-L-*N*-methylalanyl-L-*N*-methylvalyl)-*N*-(2-nitro-3-benzyloxy-4-methylbenzoyl)-L-threonyl-D-valyl-L-proline (compound 12),[12] which was converted to the *p*-nitrophenyl ester. The benzyloxycarbonyl protecting group was cleaved by 5N hydrogen bromide in dioxane. Repeated cyclization experiments under conditions used in actinomycin D synthesis, ie, at high dilution in pyridine at 60 C, failed to produce any cyclic product. Brockmann and Lackner (12) have reported failures of cyclization in attempts to synthesize several diastereoisomers of actinomycin D, and they proposed as an explanation that the open-chain intermediates were unable to assume certain conformations which are prerequisites for attaining cyclization. If one examines, with this in mind, the crystal structure of actinomycin D, determined recently by Sobell et al (46, 47) (fig 10), it appears that in the prevailing *cis* conformation of the Pro-Sar peptide bond, the β-carbon of an *N*-methylalanine residue of L configuration in position 4′ would point toward the densely packed interior of the cyclic struc-

[12] Physical constants: mp = 98–103 C; $[\alpha]_D^{20}$ -16.6° (*c* 0.33, dioxane).

ture and thus prevent cyclization by steric interference. The β-carbon of a diastereoisomer containing a D-*N*-methylalanine residue in the 4′ position would point to the outside of the cyclic structure, and ring closure might then be possible. Work in progress to examine this proposal led to preparations of D-N-methylalanine-containing peptide intermediates corresponding to compounds 10, 11, and 12.

CONCLUSIONS

It has been our aim to search for actinomycins of improved chemotherapeutic properties through chemical synthesis of peptide analogs. To be effective against tumors, actinomycins must be taken up by tumor cells (48) and retained for prolonged periods of time (49). Both uptake and retention might be influenced by changes in solubility characteristics of actinomycins which depend greatly on the peptide moieties. Thin-layer chromatographic behavior of the synthetic lactam analogs 6 and 7 (fig 6) indicated that both were, as expected, more hydrophilic than actinomycin D. Their comparative antitumor activities (table 2) indicated that the ω-methyl groups in the 1′ positions, while enhancing bacteriostatic potency in the lactones, somehow interfere with the inhibitory activity of the lactams. In fact, actinomycin D lactam (compound 7) showed only transient inhibition during the drug administration. When this ceased, tumors started growing rapidly again, which might mean that compound 7 was not taken up to the same extent as actinomycin D or that it was taken up and quickly lost from the tumor cells. For better evaluation of these synthetic analogs and other, semisynthetic derivatives and analogs, physiologic disposition studies of this kind done by Schwartz et al (49, 50) in experimental animals should be carried out, followed by clinical pharmacologic investigations. The results of such studies should provide guidelines for the design of actinomycin analogs with promising cancer chemotherapeutic properties.

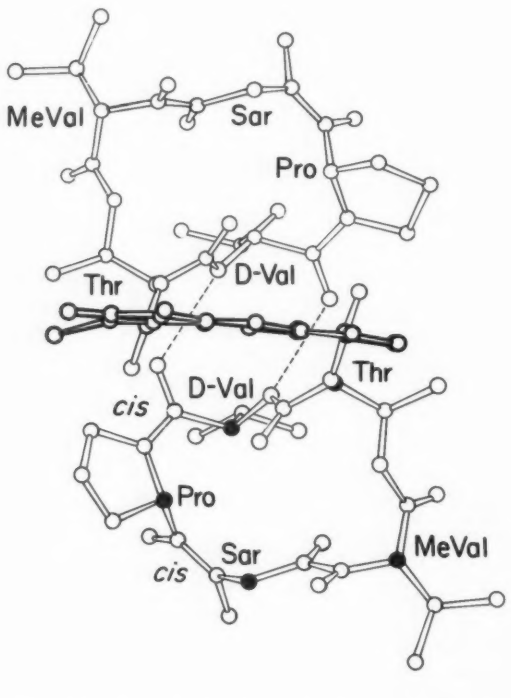


FIGURE 10.—Crystal structure of actinomycin D when complexed with two deoxyguanosine molecules (not shown). The DVal-Pro and Pro-Sar peptide bonds are in *cis* conformation. The α-carbons are shown as black circles in the lower peptide moiety. Modified from Jain and Sobell (47).

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7-Substituted Actinomycin D (NSC-3053) Analogs as Fluorescent DNA-Binding and Experimental Antitumor Agents [1,2,3]

Edward J. Modest and Sisir K. Sengupta [4,5]

SUMMARY

The synthesis of actinomycin D analogs that retain DNA-guanine binding specificity has been undertaken for two reasons: to obtain compounds that might be useful as selective fluorochromes for chromosome analysis and to obtain biologically active analogs that might exhibit qualitatively different or advantageous pharmacologic properties as growth inhibitors and antitumor agents. Two interesting analogs, 7-nitroactinomycin D and 7-aminoactinomycin D, have resulted from chemical studies on actinomycin chromophore model systems. These two compounds are comparable to, although not superior to, actinomycin D in the inhibition of mammalian (CCRF-CEM) cells in vitro and Ridgway osteogenic mouse sarcoma in vivo; however, the compounds exhibit different inhibitory profiles against two *Lactobacillus* systems in vitro. Both compounds have DNA-binding properties and DNA-guanine specificity similar to those of actinomycin D itself. 7-Aminoactinomycin D, an agent with red fluorescence, has, in addition, potential value in the analysis of mammalian cells and mammalian chromosomes. This investigation has demonstrated clearly that substitution of the actinomycin D molecule at a position that does not interfere with the DNA-binding specificity leads to biologically useful agents. Furthermore, this study indicates that other analogs with substitution at chromophore positions 7 or 8 might be pharmacologically superior to actinomycin D as antitumor agents.

[Cancer Chemother Rep 58:35–48, 1974]

Through a collaboration with Torbjörn Caspersson of the Karolinska Institute, Stockholm, Sweden, started by Sidney Farber and George E. Foley of The Children's Cancer

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[2] We thank the Lederle Laboratories Division, American Cyanamid Co., for a generous supply of actinomycin D (prepared by Dr. Nestor Bohonos and co-workers).

[3] Abbreviations for polynucleotides and their constituents follow the recommendations of the International Union of Pure and Applied Chemistry-International Union of Biochemistry (IUPAC-IUB) Commission on Biochemical Nomenclature. [*See Biochemistry* 9:4022–4027, 1970.]

[4] The Children's Cancer Research Foundation and Harvard Medical School, Boston, Mass.

[5] We acknowledge with thanks the assistance of Miss S. Karin Tinter, Miss Dorothy H. Trites, Mr. Robert D. Sitrin, Mr. Paul G. Ramsey, and Mr. Vincent P. Gibbons (chemical and biochemical determinations); Dr. George Yerganian and Miss Marilyn Nell (cytogenetic studies); Drs. George E. Foley and Herbert Lazarus (in vitro bioassays); and Miss Barbara L. Brown (experimental antitumor evaluations). The collaboration and continuing interest of Dr. Torbjörn Caspersson and his colleagues at the Karolinska Institute, Stockholm, Sweden, are gratefully acknowledged.

Research Foundation, a program to study DNA-base-specific fluorochromes that might produce selective fluorescence in chromosomal DNA was initiated (1–4).

The first proposal was to design and synthesize fluorescent analogs of actinomycin D, a compound known to be DNA-guanine specific (5,6). While the actinomycin chemistry was being worked out, quinacrine mustard dihydrochloride[6] (fig 1) (7) was selected as the first agent on the basis that it would satisfy our initial requirements for (*a*) a highly fluorescent aminoacridine nucleus that can intercalate into double-helical DNA, (*b*) basic nitrogen atoms that can form ionic bonds with DNA phosphate, and (*c*) an alkylating group in the side chain that can form covalent bonds with DNA-guanine.

Exposure of a fixed plate of *Vicia faba* metaphase chromosomes to a solution of quinacrine mustard in buffer resulted in dramatic confirmation of the thesis. Figure 2 shows the presence of three intensely fluorescent regions near the centromere of the large *V. faba* M metaphase chromosome (1,2). Even more interesting was the observation that these bands agreed in location with heterochromatin, as visualized by cold treatment and alkylating agent breakage. Furthermore, these fluorescent bands did not represent only locally intense concentrations of DNA, since a DNA scan of the chromosome by ultramicrospectrophotometry did not reveal such a correlation (2).

The further observation that each chromosome in a quinacrine mustard-stained *V. faba* metaphase plate has an individual and characteristic pattern led first to the discovery that the distal end of the long arm of human chromosome Y is intensely fluorescent (8) and then to the observation that each human metaphase chromosome has a characteristic and reproducible quinacrine mustard banding pattern that is observable both fluorometrically and visually (9–11). Both fluorescent identification banding patterns and intensely fluorescent polymorphic regions can be seen in a human chromosome metaphase plate after quinacrine mustard treatment. (A complete quinacrine mustard-stained human metaphase karyotype is shown in fig 3.) The quinacrine mustard method is now the primary international identification standard for human chromosomes (24 human chromosome types: 22 autosomes and two sex chromosomes) (12).

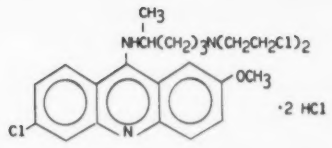


FIGURE 1.—Structure of quinacrine mustard dihydrochloride.

The original concept of the design of fluorescent actinomycin D analogs that retain DNA-guanine binding specificity then became a twofold goal: (*a*) to synthesize compounds that might be useful as selective fluorochromes for chromosome analysis and (*b*) to prepare biologically active analogs that might exhibit qualitatively different or advantageous pharmacologic properties as growth inhibitors and antitumor agents. The chemical approach was difficult since the DNA-binding properties and biologic activity of the actinomycin molecule require the intactness of the 2-amino group, the 3-quinoid oxo function, and the methyl groups at positions 4 and 6, as well as the integrity of the cyclic pentapeptide lactones. We deduced on the basis of published information (13–15) that the DNA-binding capacity and DNA-guanine specificity of the molecule could be preserved in the process of introducing a fluorescence-enhancing group by substitution at position 7 (and perhaps at position 8) of the phenoxazinone chromophore.

CHEMICAL STUDIES

The first attempt was to expand the actinomycin chromophore to a tetracyclic system by adding another aromatic ring at positions 7 and 8 in order to increase the fluorescence of the chromophore (16). One approach of this kind is to add a fluorescence-enhancing ring, such as oxazinone (17) or oxazole (18), by chemical reaction at positions 2 and 3 and then

[6] Quinacrine mustard dihydrochloride: NSC-3424; CAS reg. No. 4213-45-0; acridine, 9-[[4-[bis(2-chloroethyl)amino]-1-methylbutyl]amino]-6-chloro-2-methoxy-, dihydrochloride.

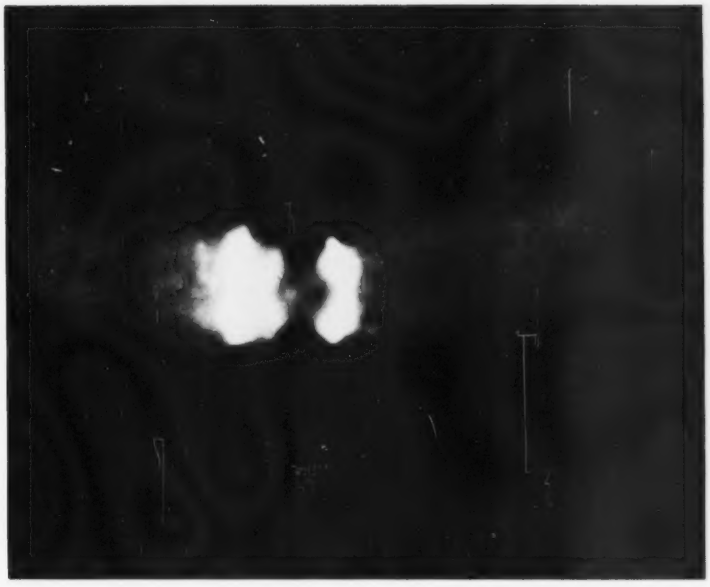


FIGURE 2.—*Vicia faba* M chromosome (quinacrine mustard stain, × 1000). From Caspersson et al (1). [Reproduced with permission from Academic Press, Inc., and T. Caspersson.]

to regenerate the essential binding functions at the original positions 7 and 8 of the new tetracyclic chromophore (fig 4). Rotation of the molecule by 180° provides a "reverse" actinomycin D analog which retains the amino and oxo binding groups, which should be fluorescent, and which should be a biochemically functional actinomycin D analog (provided there is no steric interference at position 8). Whether or not the pentapeptide lactones of this kind of "reverse" analog can rotate past each other in physiologic media is an interesting question. If they can, the molecule should have the normal properties of actinomycin D; if not, an interesting isomeric analog of actinomycin D would result.

In order to conserve our limited supply of actinomycin D, we decided to synthesize actinomycin chromophore model compounds (fig 5) in which R_1 and R_2 are both ethyl groups or *n*-propyl and hydrogen. The structures shown in figure 6 are new compounds prepared on the basis of earlier work (19,20) from the starting material 3-benzyloxy-2-nitro-*p*-toluic acid (20). A number of actinomycin chromophore analogs have been prepared, and the work has been extended to the preparation of three analogs of actinomycin D: the 7-hydroxy, 7-nitro, and 7-amino compounds. Hans Brockmann and coworkers have reported (17) the corresponding analogs of actinomycin C_3[7] prepared by somewhat different methods.

Reduction of the bis(diethylamide) model compound and reaction with pyruvate (16) gave a tetracyclic oxazinone (fig 6). Oxidation with 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) gave the 7-oxo compound, and cleavage of the oxazinone protecting group with silica gel at pH 7.2 led to the model 7-hydroxy compound in one step. Application of this reaction scheme to actinomycin D provided 7-hydroxyactinomycin D.

Nitration of the 7-hydroxy model compound gave the 7-hydroxy-8-nitro analog. Reduction of this analog provided an interesting symmetrical compound with binding groups on both ends of the ring system (fig 7). Condensation of the latter compound with pyruvate under var-

[7] Actinomycin C_3: NSC-87222; CAS reg. No. 6156-47-4.

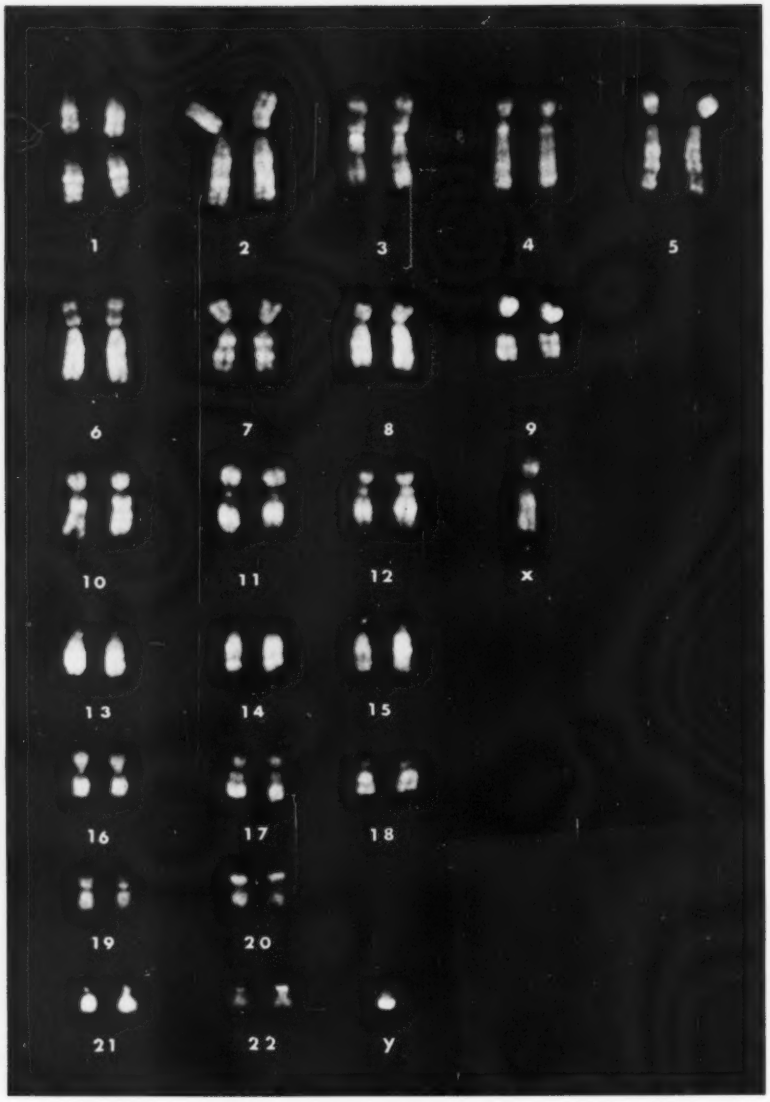
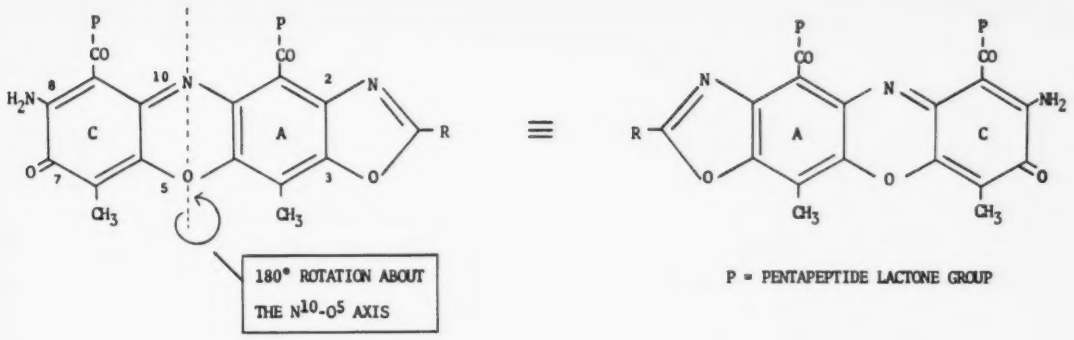
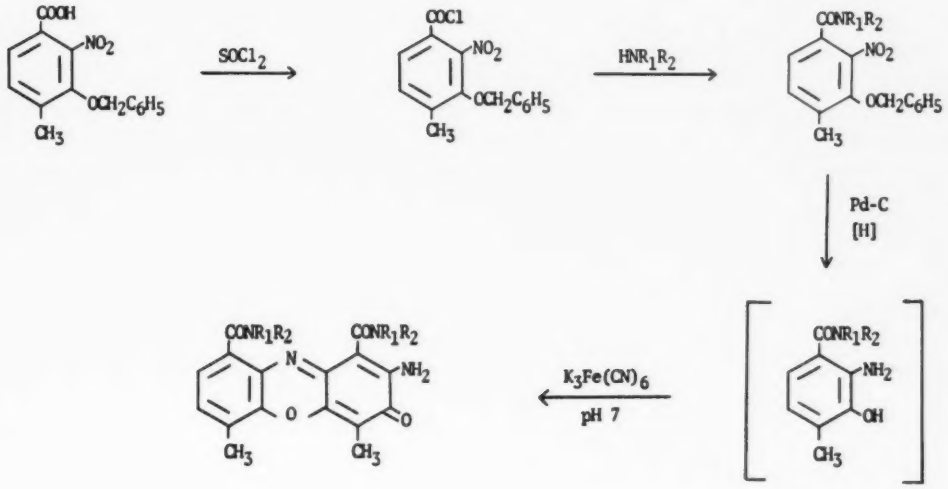


FIGURE 3.—Human metaphase karyotype (quinacrine mustard stain, × 2500). From Caspersson et al (10). [Reproduced with permission from Academic Press, Inc., and T. Caspersson.]



BY INCORPORATION OF THE BINDING GROUPS AT POSITIONS 2 AND 3 OF THE ACTINOMYCIN MOLECULE INTO A FLUORESCENCE-ENHANCING RING AND REGENERATION OF THE SAME BINDING GROUPS AT POSITIONS 7 AND 8, A FLUORESCENT ACTINOMYCIN DERIVATIVE WITH COMPARABLE DNA-BINDING PROPERTIES SHOULD RESULT.

FIGURE 4.—Proposed reversal of rings A and C of the actinomycin molecule by synthetic modification.



$R_1 = R_2 = C_2H_5$

$R_1 = H$; $R_2 = \underline{n}\text{-}C_3H_7$

FIGURE 5.—Synthesis of the actinomycin chromophore model compounds.

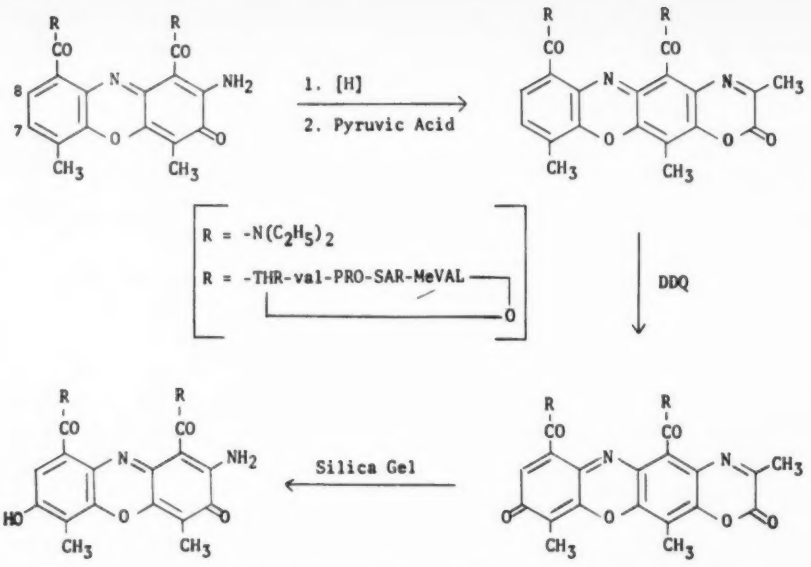


FIGURE 6.—Synthesis of 7-hydroxyactinomycin D and related model compounds.

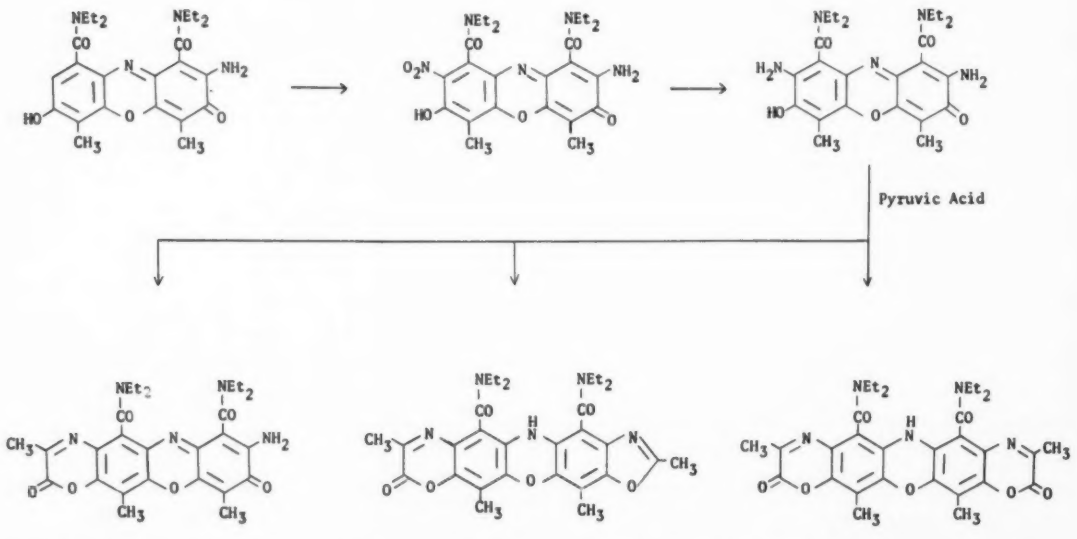


FIGURE 7.—Tetracyclic and pentacyclic actinomycin chromophore analogs from the 7-hydroxyactinomycin chromophore model compound.

ied conditions provided a separable mixture of three compounds with oxazinone and oxazole rings: two pentacyclic compounds and one tetracyclic compound. The pentacyclic analogs of actinomycin D should not bind to DNA, but selective removal of the oxazinone ring at slightly alkaline pH or of the oxazole ring at acid pH should lead to actinomycin D analogs with functional 2-amino and 3-oxo binding groups.

Reaction of the 7-hydroxy-8-nitro analog with pyruvate under reflux gave the oxazole

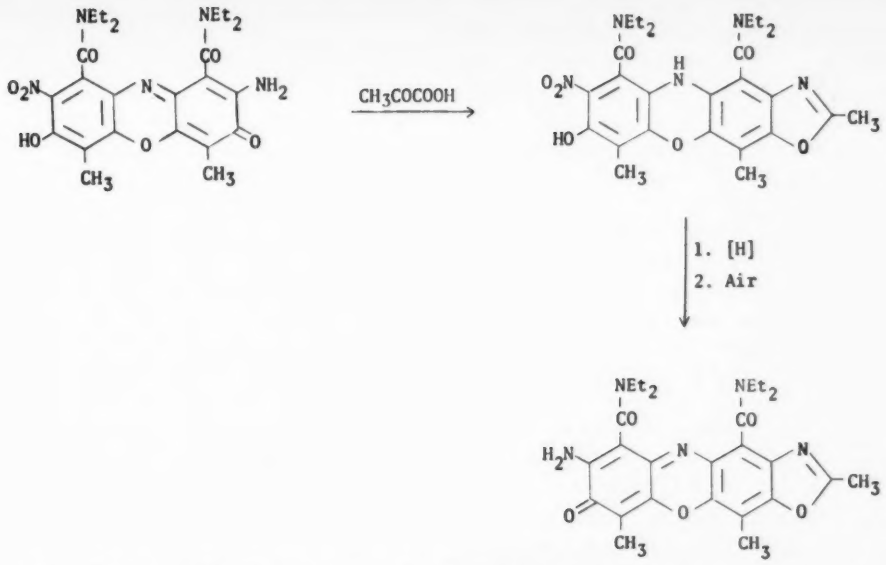


FIGURE 8.—Synthesis of the reverse oxazole analog of the actinomycin chromophore model compound.

analog (fig 8). This simple method proved to be superior to the earlier, less consistent procedure involving reduction and reaction with acetaldehyde (18). Reduction of the nitro group in this tetracyclic chromophore analog then restored the amino and oxo binding groups.

Figure 9 shows the direct nitration of the bis(diethylamide) chromophore model compound to the 7-nitro analog with sulfuric/nitric acids and without protection of the 2-amino group. Catalytic reduction afforded the 7-amino compound. Application of these reactions to actinomycin D gave the 7-nitro and 7-amino analogs of the antibiotic (21).

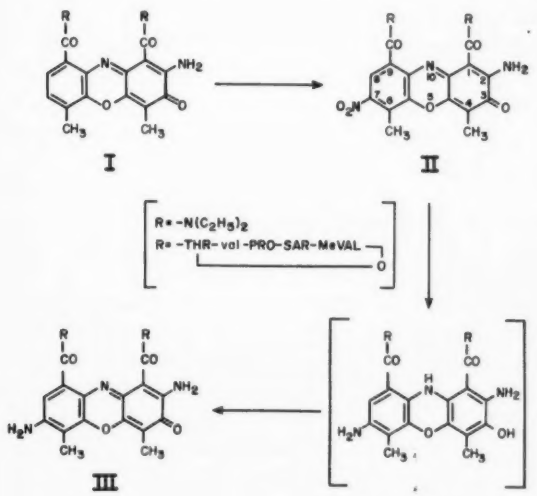


FIGURE 9.—Synthesis of 7-nitro and 7-amino analogs of actinomycin D and the chromophore model compound. From Modest and Sengupta (23). [Reproduced with permission from Academic Press, Inc.]

BIOPHYSICAL AND FLUORESCENCE STUDIES

The first biochemical experiments were designed to prove whether these 7-substituted actinomycin D analogs possessed the G · C base pair-specific binding functions of the parent antibiotic. This was done in several ways. Melting temperature experiments with calf thymus DNA (fig 10) indicated that actinomycin D, 7-nitroactinomycin D, and 7-aminoactinomycin D all give the same melting temperature elevation of +11.5 C, the three curves being superimposable. 7-Bromoactinomycin D, a gift from Henry Sobell (The University of Rochester, Rochester, NY) (6), binds even more strongly with a temperature increment of +13 C.

The first evidence that both 7-nitroactinomycin D and 7-aminoactinomycin D have G · C base pair binding specificity was provided (fig 11) by difference spectral measurements (5) in which the degree of DNA-base-specific binding can be estimated by optical density changes plotted as a function of wavelength. Binding is evident for actinomycin D, 7-nitroactinomycin D, and 7-aminoactinomycin D with calf thymus DNA but not with poly(dA-dT).

Scatchard plots based on ultrafiltration experiments with the Paulus apparatus (22) at various concentration ratios of calf thymus DNA to either actinomycin D or 7-aminoactinomycin D (fig 12) indicate that the two compounds have comparable binding constants and nucleotide:ligand binding ratios (21,23) and that these values compare favorably with those determined by Donald Crothers (24) via equilibrium dialysis for actinomycin D, actinomycin C_3, and 7-aminoactinomycin C_3.

In summary, 7-nitroactinomycin D and 7-aminoactinomycin D have been shown by sev-

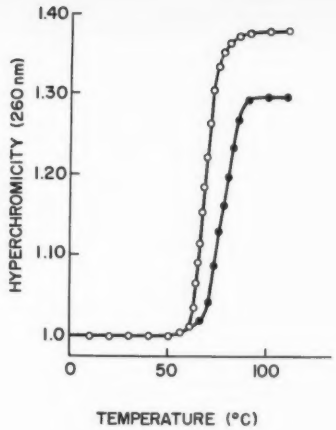


FIGURE 10.—Thermal denaturation profile of calf thymus DNA in the absence (○——○) and in the presence (●——●) of actinomycin D, 7-nitroactinomycin D, and 7-aminoactinomycin D (coincident curves). Measurements were done in 0.01 M phosphate buffer at pH 7.0. From Modest and Sengupta (23). [Reproduced with permission from Academic Press, Inc.]

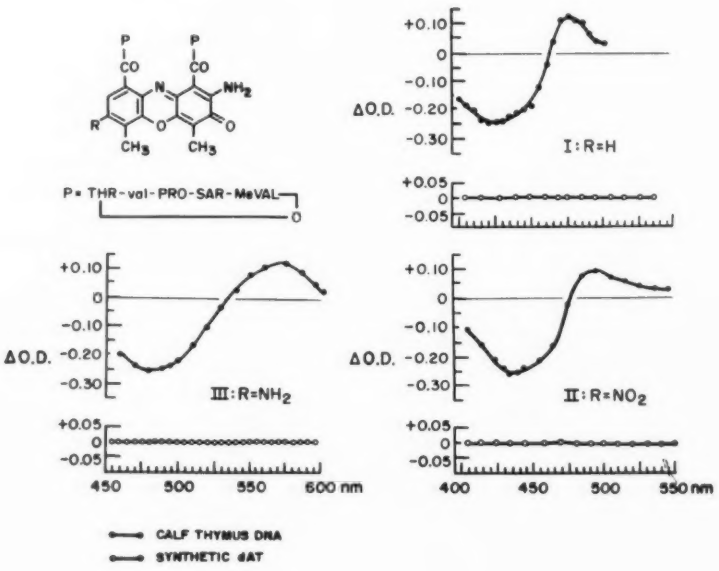


FIGURE 11.—Complex formation between actinomycin D (I), 7-nitroactinomycin D (II), and 7-aminoactinomycin D (III) and calf thymus DNA (●——●) or poly (dA-dT) (○——○) as determined by difference spectra. Measurements were done in 0.01 M phosphate buffer at pH 7.0.

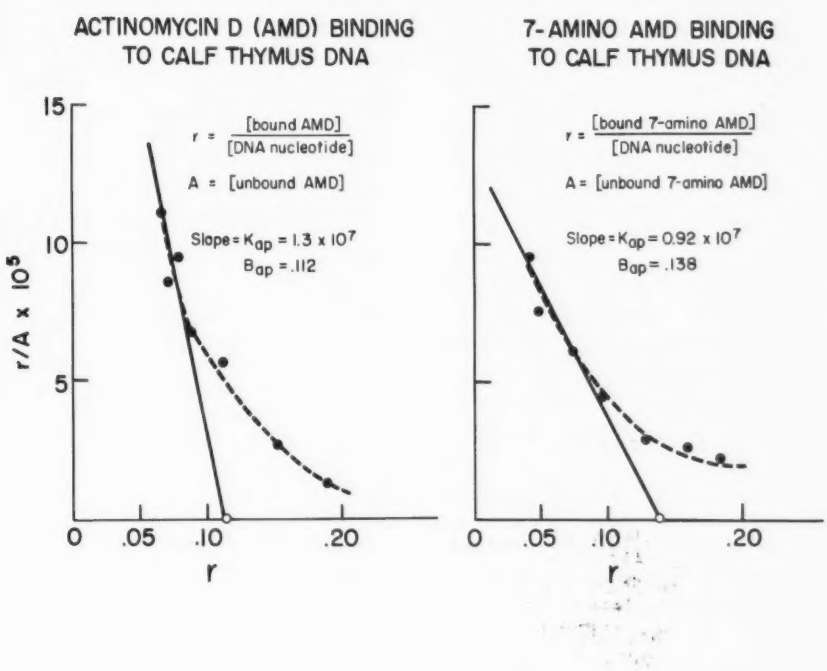


FIGURE 12.—Scatchard plots (by ultrafiltration) for binding of actinomycin D and 7-aminoactinomycin D to calf thymus DNA in 0.01 M phosphate buffer at pH 7.0. K_{ap} = apparent binding constant in moles^{-1}; B_{ap} = apparent number of binding sites per nucleotide.

eral methods to bind to DNA with G • C specificity in a manner qualitatively and quantitatively similar to that of actinomycin D. Studies on the inhibition of RNA polymerase by these analogs are in progress.

7-Aminoactinomycin D proved to be a highly fluorescent compound with an excitation maximum at 465 nm and an emission peak in the red at 595 nm in aqueous media (21,23,25). Measurements in vitro were more efficient at the excitation maximum of the 7-aminoactinomycin D-DNA complex, 540 nm. Studies of this kind based on work done previously by our laboratory (26) and by Bernard Weisblum (27) and Rudolf Rigler (28,29) on the fluorescence of aminoacridine-DNA complexes were carried out. The fluorescence in aqueous solution of complexes of polynucleotides with aminoacridine intercalating agents (such as quinacrine mustard, quinacrine, and proflavine) is enhanced by A • T base pairs in polydeoxynucleotides and quenched by polynucleotide guanine (23,27–29).

Figure 13 shows variation of the relative fluorescence of 7-aminoactinomycin D with increasing polynucleotide:fluorochrome ratios (23,25). No change in fluorescence and, therefore, no binding occurs for poly (dA-dT), poly (rG), or poly (rA), in accordance with the absolute requirement for G • C base pairs in bihelical DNA for binding of actinomycin D. When 7-aminoactinomycin D binds, fluorescence is enhanced, but the enhancement is *inversely* proportional to the G + C content of the polynucleotides, decreasing in the following order: calf thymus DNA (42% G +

C), *Micrococcus lysodeikticus* DNA (72% G + C), and poly (dG) · poly(dC) (100% G + C).

This work has been extended to an examination of the fluorescence behavior of 7-aminoactinomycin D in the presence of deoxynucleosides. In figure 14, it can be seen that at somewhat higher nucleoside:fluorochrome ratios the fluorescence of this compound is enhanced by 2′-deoxyadenosine, quenched by 2′-deoxyguanosine, and unaffected by 2′-deoxycytidine.

On the basis of the observations presented in figures 13 and 14, we conclude that the binding specificity of this fluorescent actinomycin analog for deoxyguanosine is evident and that it results in a quenching of fluorescence, but that the fluorescence of the complex is enhanced by A · T base pairs near the binding site. This approach offers a unique method for evaluating the binding of actinomycin analogs to DNA and for probing the nature of the binding site environment.

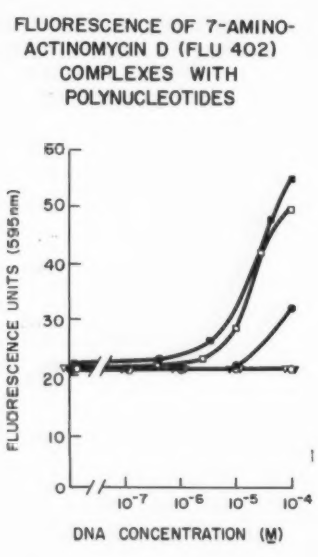


FIGURE 13.—Effect of polynucleotides on 7-aminoactinomycin D fluorescence in 0.1 M phosphate buffer at pH 6.8. Concentration of 7-aminoactinomycin D = 5×10^{-6} M. Excitation wavelength = 540 nm. ■——■ = calf thymus DNA (42% G + C); □——□ = *Micrococcus lysodeikticus* DNA (72% G +C); ●——● = poly(dG) • poly(dC) (100% G + C); ○——○ = poly(dA-dT); ▼——▼ = poly(rG); ▽——▽ = poly(rA). From Modest and Sengupta (23). [Reproduced with permission from Academic Press, Inc.]

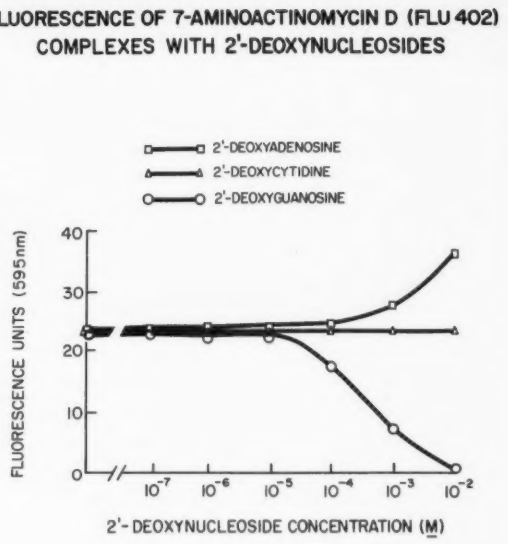


FIGURE 14.—Fluorescence of 7-aminoactinomycin D complexes with 2′-deoxynucleosides in 0.1 M phosphate buffer at pH 6.8. Concentration of 7-aminoactinomycin D = 5×10^{-6} M. Excitation wavelength = 540 nm. □——□ = 2′-Deoxyadenosine; △——△ = 2′-deoxycytidine; ○——○ = 2′-deoxyguanosine.

A mechanism for the quenching of fluorescence of aminoacridine fluorochrome-polynucleotide complexes via charge transfer complex formation with DNA-guanine has been proposed (30,31); this explanation may be applicable to actinomycin fluorochromes as well. A mechanism for fluorescence enhancement is less obvious.

Thus, a possible deoxynucleotide sequence for the binding of a fluorescent actinomycin D analog to bihelical DNA with enhancement of fluorescence would involve adjacent G · C base pairs for binding surrounded by A · T base pairs for enhancement. In this model system, the chromophore of the actinomycin D analog would intercalate between adjacent G · C base pairs, and fluorescence behavior would be influenced by electronic effects in-

FIGURE 15.—Red fluorescent visualization of DNA segments in *Drosophila melanogaster* polytene chromosomes by 7-aminoactinomycin D (× 1000 before reduction).

volving the G • C base pair binding site and the neighboring A • T base pairs.

It is of interest that the association of actinomycin D with deoxyguanosine (6) has been confirmed by our relatively simple deoxynucleoside fluorescence-enhancement experiments (fig 14).

Initial efforts to use 7-aminoactinomycin D as a fluorochrome for chromosome analysis have shown that DNA segments of *Drosophila melanogaster* polytene chromosomes are stained with a bright-red fluorescence with this compound (fig 15) (32). Further work is planned with smaller chromosomes (including human metaphase chromosomes) at an excitation wavelength of 540 nm, the optimal excitation for the 7-aminoactinomycin D-DNA complex. These studies require special microfluorometric instrumentation and will involve Torbjörn Caspersson and his associates in Stockholm.

BIOASSAY DATA

The 7-substituted actinomycins have been investigated for biologic inhibitory properties in several systems at The Children's Cancer Research Foundation. Inhibitory in vitro data are presented in table 1. In both the *Lactobacillus fermenti*-thiamine and the *Lactobacillus arabinosus*-pantothenate systems (33), 7-nitroactinomycin D is approximately as active as actinomycin D. However, 7-aminoactinomycin D is only about one tenth as active as actinomycin D in the *L. fermenti*-thiamine system and is essentially inactive in the *L. arabinosus*-pantothenate system. In mammalian cell culture assay (34), all three compounds show comparable activity.

Data on tumor inhibition by the three compounds are given in tables 2 and 3. Chemotherapeutic activity was evaluated against the Ridgway osteogenic sarcoma implanted intramuscularly (im) in AKD_2/F_1 mice (35). When the tumor was palpable (17 days after implantation), compounds were injected intraperitoneally (ip) for 4 successive days. Twelve mice were used for each dose level. When the

TABLE 1.—In vitro inhibitory activity of actinomycin D, 7-nitroactinomycin D, and 7-aminoactinomycin D

		ID50 (μg/ml)		
Compound	*System:*	*L. arabinosus*-pantothenate (0.01 μg/ml)	*L. fermenti*-thiamine (0.01 μg/ml)	CCRF-CEM cell line
Actinomycin D		0.008	0.006	0.065
7-Nitroactinomycin D		0.007	0.0075	0.080
7-Aminoactinomycin D		0.3	0.072	0.085

TABLE 2.—Tumor inhibition by actinomycin D, 7-nitroactinomycin D, and 7-aminoactinomycin D*

Compound	Tumor inhibition in animals treated with—					
	37.5 μg/kg/day		75 μg/kg/day		150 μg/kg/day	
	Tumor weight† (g)	T/C‡	Tumor weight† (g)	T/C‡	Tumor weight† (g)	T/C‡
Untreated controls	13.8	—	13.8	—	13.8	—
Actinomycin D	4.2	0.30	0.1	0.007	0.3	0.022
7-Nitroactinomycin D	12.9	0.93	10.7	0.77	0.2	0.014
7-Aminoactinomycin D	13.1	0.95	1.0	0.072	0.5	0.036

*Compounds were given ip daily on 4 successive days (starting on Day 17 after tumor implantation) after visualization of the im implanted Ridgway osteogenic sarcoma in AKD_2/F_1 mice. There were six mice in each group. Mice were sacrificed when the first untreated control animal died (Day 15 after therapy started).

†Averaged wet weights of excised tumors.

‡T/C = treated animal tumor weight/control animal tumor weight. Toxicity was observed with all agents after a dose of 300 μg/kg/day for 4 successive days.

first untreated control animal died on Day 15 after the first injection (or Day 32 after tumor implantation), six animals at each dose level were killed and the tumors were excised, weighed, and compared (table 2). At the 150-μg/kg/day dose level, all three compounds were comparable in effectiveness, with somewhat better results being obtained for actinomycin D and 7-nitroactinomycin D. At the 75-μg/kg/day dose level, actinomycin D was superior and 7-aminoactinomycin D showed some activity. At the 37.5-μg/kg/day dose level, only actinomycin D was inhibitory.

The remaining six mice at each dose level were evaluated for long-term survival (table 3). At the 150-μg/kg/day dose level, all three compounds produced long-term survivors without detectable tumor: on Day 185 after the start of therapy, four of six mice treated with actinomycin D, three of six mice treated with 7-nitroactinomycin D, and two of six mice treated with 7-aminoactinomycin D were alive. These survivors were killed on Day 185 after the start of therapy. Median survival times are given in table 3. No tumors were found at autopsy in the surviving mice and, presumably,

TABLE 3.—Survival time of tumor-bearing mice treated with actinomycin D, 7-nitroactinomycin D, and 7-aminoactinomycin D*

Compound	Survival time in animals treated with—					
	37.5 μg/kg/day		75 μg/kg/day		150 μg/kg/day	
	MST† (days)	% ILS‡	MST† (days)	% ILS‡	MST† (days)	% ILS‡
Untreated controls	16.5	—	16.5	—	16.5	—
Actinomycin D	29.5	79	50.0	203	>185	>1020§
7-Nitroactinomycin D	18.5	+12	14.5	−12	>135	>718¶
7-Aminoactinomycin D	14.5	−12	35.5	115	103	524**

*Compounds were given ip daily on 4 successive days after visualization of the im implanted Ridgway osteogenic sarcoma in AKD_2/F_1 mice. There were six mice in each group.

†MST = median survival time.

‡% ILS = percent increase in lifespan of treated mice compared with control mice, calculated from the first day of therapy (Day 17 after tumor implantation). Toxicity was observed with all agents after a dose of 300 μg/kg/day for 4 successive days.

§Four of six mice still alive and without palpable tumor when sacrificed on Day 185 after the start of therapy.

¶Three of six mice still alive and without palpable tumor on Day 185 after the start of therapy.

**Two of six mice still alive and without palpable tumor on Day 185 after the start of therapy.

the survivors would have lived substantially longer without tumor. At the lower dose levels tested in this experiment, actinomycin D was more effective than the two analogs.

The Ridgway osteogenic sarcoma is clearly sensitive to actinomycin D and the two analogs reported in this paper. Further experiments with the Ridgway tumor (tested under more challenging conditions) and other mouse tumors (including P388 lymphatic leukemia and S91 melanoma) are in progress.

Analysis of the biologic data makes it clear (*a*) that these two 7-substituted analogs of actinomycin D are comparable to, although not superior to, actinomycin D in one transplantable mouse tumor system; (*b*) that all three compounds are comparable in mammalian cell culture assay in vitro; and (*c*) that the antibacterial assay systems used (14,17,33) do not necessarily provide good predictive data for other bioassay systems.

Overall, the most important aspect of this investigation is that we have established that substitution of the actinomycin D molecule at position 7 provides analogs with biochemical activity, DNA-binding properties, DNA-binding specificity, inhibitory properties in vitro, and experimental antitumor activity in vivo comparable to the corresponding properties of actinomycin D itself.

With respect to the first objective of this study, we have demonstrated that a fluorescent analog of actinomycin D with the desired DNA-binding properties can be synthesized and that this compound or related compounds may be useful for the analysis of mammalian chromosomes and the mammalian cell cycle.

As for the second objective, it is clear that the antitumor activity of the actinomycin D molecule can be retained in 7-substituted analogs. It is not important at this stage that 7-nitroactinomycin D or 7-aminoactinomycin D be decidedly superior to actinomycin D in antitumor or in vitro cytotoxic activity, but it is important that effective experimental antitumor agents can be prepared by substitution of actinomycin D at positions that do not interfere with the DNA-binding specificity of the molecule. The importance of this conclusion can be understood by consideration of the lack of DNA-binding properties and in vitro biologic activity of actinomycin D analogs in which even the most minor chemical changes at positions other than 7 or 8 have been introduced (14,24,36).

Continuation of this type of research may well lead to more effective actinomycin D analogs with improved therapeutic indices, to analogs with altered and more favorable transport properties (by introduction of lipophilic substituents at position 7), or to covalent macromolecular complexes of actinomycin D (by covalent bonding at position 7).

7-Nitroactinomycin D and 7-aminoactinomycin D are the most active synthetic analogs of actinomycin with respect to experimental antitumor activity. We believe that the results of this investigation are significant and suggestive of profitable extension in experimental cancer chemotherapy.

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The Clinical Use of Actinomycin [1]

Emil Frei III [2]

SUMMARY

The actinomycins have shown important antitumor activity in man. Actinomycin D alone or in combination with methotrexate will cure 50%–70% of patients with gestational choriocarcinoma. In Wilms' tumor actinomycin D produces transient tumor regression in 40% of patients with advanced disease. In primary Wilms' tumor the cure rate is increased from 40% to 80% when actinomycin D is used in multiple courses in combination with surgery and radiotherapy. In embryonal rhabdomyosarcoma, Ewing's sarcoma, and other childhood tumors, actinomycin D alone, and particularly in combination, is effective. Also, there is suggestive evidence that for Ewing's sarcoma and rhabdomyosarcoma the cure rate from primary treatment is increased by the concurrent use of combination chemotherapy including actinomycin D. Actinomycin D alone or in combination will produce tumor regression in 20%–60% of patients with testicular tumors, and 5%–10% of patients with embryonal carcinoma of the testis will have long-term tumor-free survival. The actinomycins are also active in patients with Hodgkin's and non-Hodgkin's lymphoma, but further studies are required to define this activity more precisely. Actinomycin D has been adequately studied in patients with metastatic breast cancer, gastrointestinal cancer, lung cancer, hypernephromas, and head and neck cancer, and has been observed to be inactive. Activity, however, has been demonstrated in patients with melanomas and sarcomas and further studies are indicated. Actinomycin D alone or in combination with vincristine produces a high response rate in Kaposi's sarcoma in Africa. Thus, actinomycin D warrants further studies in certain tumors and additional studies of dose schedule, combination chemotherapy, and selected congeners.

[Cancer Chemother Rep 58:49–54, 1974]

Actinomycin D is one of the most important clinical antitumor agents. It is capable of curing gestational choriocarcinoma, and combined with local therapy it is also capable of increasing the cure rate in Wilms' tumor. Its spectrum of antitumor activity in man has been considered to be relatively narrow, but this observation deserves re-examination for several reasons including: (*a*) most of the phase II studies were performed in the 1950s prior to the development of modern therapeutic trial techniques; (*b*) many of the trials were performed with impure materials or mixtures; and (*c*) since its introduction into the clinic

[1] Chemical nomenclature, NSC numbers, and CAS registry numbers for all compounds mentioned in this paper are included in the List of Compounds at the end of the text.

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in the mid 1950s new roles for actinomycin D have been found such as in choriocarcinoma (1962) and Kaposi's sarcoma (1972).

Choriocarcinoma

It was demonstrated in 1957 by Li, Hertz, and Spencer that methotrexate when administered in intermittent intensive courses was curative in a substantial number of patients. The response and cure rate correlated inversely with the extent of disease. Thus, the height of the human chorionic gonadotropin titer (HCG) and the extent of the disease in terms of prior duration of symptoms adversely affected prognosis. By this definition, for those patients with extensive disease the curative rate was only 40%–50% (1). A number of treatment programs were studied in the relapsing patients. By far the most active compound was actinomycin D. As with methotrexate, cures were achieved when actinomycin D was given in 5–7-day courses intermittently. In choriocarcinoma, the HCG level correlates closely with the number of neoplastic cells. In fact, it has been estimated that as few as 1000 persisting neoplastic cells can be detected by HCG assay. Thus, for methotrexate and actinomycin D, treatment was continued until the hormone titer normalized, after which an additional three or four courses of treatment were given. Actinomycin D is now commonly used as the initial treatment for patients with gestational choriocarcinoma and has the advantage over methotrexate of not being hepatotoxic. There is some evidence that actinomycin D plus methotrexate given either concurrently or sequentially may further increase the cure rate (1).

Wilms' Tumor

Shortly after its introduction into the clinic, actinomycin D was found to be capable of producing objective tumor regression in approximately 40% of patients with advanced metastatic Wilms' tumor (2). However, in all patients relapse occurred within a few months, and re-treatment was less effective or ineffective.

At that time, the primary treatment for Wilms' tumor limited to the abdomen consisted of surgery plus radiotherapy. This treatment produced a cure rate of approximately 40% depending upon the stage of the disease within the abdomen and the age of the patient. For most tumors, there is a period after treatment where, if patients remain relapse-free, they have a high probability (> 90%) of being cured. For Wilms' tumor this interval is 2 years, and for choriocarcinoma it is 1 year. Since actinomycin D was effective though not curative in patients with advanced Wilms' tumor, Dr. Farber added actinomycin D to radiotherapy plus surgery in the primary treatment of the disease in an effort to increase the cure rate. In general, courses of treatment were given for a total of 6–18 months. This program essentially doubled the cure rate (to 80+%) (3). The importance of giving multiple courses of treatment over a 15-month period rather than giving a single course of treatment was demonstrated in a randomized, comparative study where the 2-year relapse-free rate was 48% after a single course and 68% after multiple courses. Recurrent metastatic Wilms' tumor almost invariably and often exclusively involves the lung. Re-treatment of metastatic disease with radiotherapy plus actinomycin D, particularly in those patients who have had no prior actinomycin D or only a single course of actinomycin D, is often highly successful and probably curative. Because of this, the overall survival rate for patients initially treated with multiple courses of actinomycin D was only slightly superior to the survival rate in those patients who received a single course (4).

This use of actinomycin D as initial therapy combined with surgery and radiotherapy was the first demonstration that adjuvant chemotherapy could be effective in increasing the cure rate for Wilms' tumor. There is substantial clinical, cytokinetic, and experimental evidence that chemotherapy is much more effective against microfoci of cancer than against more advanced metastatic disease. The theoretical basis, importance, and promise of adjuvant chemotherapy has been emphasized (5).

Embryonal Rhabdomyosarcoma

Embryonal rhabdomyosarcoma is a rare childhood solid tumor. Actinomycin D produces objective tumor regression in approximately

30% of such patients (2). Actinomycin D is commonly employed as systemic treatment combined with surgery and/or radiotherapy for patients with localized forms of embryonal rhabdomyosarcoma. There is suggestive evidence, based on comparisons with historical controls, that such treatment may increase the cure rate.

Testicular Tumors

Objective responses to actinomycin D in patients with metastatic testicular cancer have been demonstrated. Responses occur in 20%–60% of patients with metastatic seminoma, embryonal cell carcinoma of the testis, and testicular and other nongestational choriocarcinomas. Dr. Li combined actinomycin D with a folic acid antagonist and an alkylating agent. With this triple combination, a high initial response rate was achieved in metastatic carcinoma of the testis, and perhaps 5%–10% of patients with embryonal carcinoma of the testis had long-term tumor-free survival. There is evidence that actinomycin D is the most important component in this combination, and indeed, other studies have suggested that actinomycin D administered in full doses is as effective as combination chemotherapy for testicular neoplasms. More recent studies have included the combinations of actinomycin D, bleomycin, and vincristine (2,6).

More recently, actinomycin D has been employed in combination with surgery and/or radiotherapy for the initial treatment of patients that have embryonal cell carcinoma of the testis. While the overall results of some of these studies are promising, the number of patients and the period of followup are at present inadequate for definitive evaluation.

Ewing's Sarcoma

Actinomycin D is effective in the treatment of metastatic Ewing's sarcoma and is currently being employed in combination with cyclophosphamide and vincristine and with radiotherapy in the primary treatment of patients with Ewing's sarcoma (7).

Lymphoma

In published studies in the 1950s, actinomycin D produced objective tumor regression in 40%–50% of patients with Hodgkin's and non-Hodgkin's lymphoma (2). It is of interest that further reported studies of actinomycin D in lymphoma have not been forthcoming. This may have resulted from the fact that negative studies tend not to be reported or that there are a number of other effective agents for lymphoma which have complicated or precluded further trials with actinomycin D. In a recent study, actinomycin D was found to be marginally effective in prolonging the duration of

TABLE 1.—Effect of actinomycin D in various tumor categories*

Metastatic tumor	No. of evaluable patients	No. of objective responses	Response (%)
Breast	45	5	11
Colorectal	48	5	15
Lung	16	0	0
Melanoma	58	19	35
Kidney, adenocarcinoma	7	0	0
Adult sarcoma	62	14	23
Head and neck	11	0	0
Pancreas	4	0	0

*Collected data from the English literature (*from* Livingston and Carter, ref 2).

MOPP-induced complete remissions in patients with Hodgkin's disease (8).

Other Tumors

Phase II studies in the English literature, as collected by Livingston and Carter (2), are presented in table 1. It seems reasonably safe to conclude that actinomycin D employed in 5–7-day courses every 3–4 weeks is inactive in patients with metastatic breast cancer, gastrointestinal cancer, lung cancer, hypernephromas, and head and neck cancer. A response rate of 35% was achieved in patients with metastatic melanoma. This response was a composite of four studies where the response rate ranged from 0 to 66% (2). Clearly, further studies in melanoma are indicated. A response rate of 23% was achieved in adults with soft tissue sarcomas. Adriamycin, a somewhat related drug, produces a response rate of 30% in this group of tumors.

Actinomycin D, usually in combination, continues to be used in several neoplastic diseases. Actinomycin D combined with NSC–45388 and BCNU has produced a response rate of 19% in patients with melanoma. It is under study as a non-cell-cycle-specific agent in a complex therapeutic program for lung cancer. It has been used as reinforcement therapy in patients with acute lymphocytic leukemia in remission and in combination with an alkylating agent and 5-fluorouracil in ovarian cancer. Most of these studies are relatively recent and it is questionable whether actinomycin D is in fact effective in these circumstances.

Kaposi's Sarcoma

Kaposi's sarcoma is a common disease in certain parts of Africa. Actinomycin D was found to produce objective response in nine of ten patients (four complete remissions) with Kaposi's sarcoma, and when it was combined with vincristine, ten of 14 patients achieved a complete remission.[3] Thus, actinomycin D is highly effective in the initial treatment of this disease. Its long-term effect on the disease requires further followup studies.

Toxicity

The toxicity of actinomycin D is presented in table 2. Actinomycin D must be given intravenously into a running infusion. Paravenous infiltration results in local necrosis. Actinomycin D produces nausea, vomiting, and malaise beginning several hours after treatment and lasting for as long as 1 day. These effects can be modified by antiemetic and sedative therapy suggesting that they are of central origin. The major and dose-limiting toxicity of actinomycin D relates to myelosuppression. Actinomycin D produces a decrease in cellularity of the bone marrow by 3–4 days after a course of treatment and depression of the peripheral white blood cell count and usually the platelet count by 8–15 days. Mucosal ulcerations and ulcerative lesions of the gastrointestinal tract associated with pain and diarrhea may occur in 30% of patients. Bone marrow depression particularly, and the mucosal and gastrointestinal ulcerations as well, are dose dependent and can be managed by proper adjustment of dose and schedule. Certain skin manifestations have been described in a minority of patients receiving actinomycin D. The active hair follicle is damaged by actinomycin D so that reversible alopecia is common. Immunosuppression occurs with actinomycin D, and indeed the drug has been employed as an immunosuppressant for organ transplantation.

The interaction of actinomycin D with radiotherapy is of interest. This was first noted in the clinic where erythema and more extensive skin reactions were found to occur in previously irradiated sites when patients received actinomycin D. Radiotherapy given after actinomycin D therapy did not produce this effect. In prospective studies it has been demonstrated that 500–600 rads are required to produce skin erythema; in the presence of actinomycin D, however, as few as 100 rads will produce such erythema.

Dose and Schedule

The dose and schedule of actinomycin D employed in man are presented in table 2.

[3] Vogel CL, et al. Treatment of Kaposi's sarcoma with a combination of actinomycin D and vincristine: results of a randomized clinical trial. Submitted for publication.

TABLE 2.—Actinomycin D: dose schedule, route of administration, and toxicity

	Dose schedule (initial dose)	
	By weight	By body-surface area
Children	15 μg/kg iv, qd × 5	450 mg/m², qd × 5
Adults	10 μg/kg iv, qd × 5 (may be repeated at 3–8-wk intervals, or weekly injections of 1–2 mg may be given)	450 mg/m², qd × 5

Route

Iv; isolation-perfusion; extravasation produces major local toxicity

Toxicity

a. Nausea, vomiting, malaise for 4–20 hrs; central origin
b. Bone marrow: stem and replicative cell damage, hypoplasia, leukopenia, and thrombocytopenia (dose limiting); may be protracted due to stem cell damage
c. Oral lesions: ulcerations
d. Gastrointestinal lesions: ulcerations, pain, and diarrhea
e. Skin: acneiform lesions, erythema, increased pigmentation
f. Alopecia
g. Immunosuppression
h. Exacerbation of skin reaction in irritated areas (items *c*, *d*, *e*, *f*); may extend to deeper tissues

Actinomycin D was initially studied in children who received 5–7-day courses consisting of 60–70 μg/kg total dose. This dose was found to produce excessive toxicity in adults. This toxicity plus the variation in different lots of the actinomycins resulted in the compounds having a reputation of producing variable and often excessive toxicity in adults. In recent years it has been found that surface area is a much more effective and physiologically accurate unit for dosing than weight. On a surface-area basis the tolerated dose for actinomycin D in children and adults is comparable (table 2).

Schedule of drug administration.—While there is comparatively little evidence for schedule sensitivity in experimental systems, it should be emphasized that actinomycin D in man has, with only one or two exceptions, been given in 5–7-day courses intermittently. A more definitive exploration of the effect of dose schedule in experimental systems and in man seems indicated. Recent studies concerning the cytokinetic effects of the drug alone or in combination support this position.

In summary, actinomycin D is an important, clinically useful, antitumor agent. It is demonstrably effective in a number of tumors and there are additional tumor categories such as melanoma, lymphoma, and soft tissue sarcomas where its activity remains to be defined. More definitive exploration of dose schedules, combination chemotherapy, and perhaps, most particularly, clinical study of selected congeners of actinomycin D are indicated.

LIST OF COMPOUNDS

Actinomycin D: NSC-3053
Adriamycin: NSC-123127
BCNU: NSC-409962; CAS reg. No. 154-93-8; urea, 1,3-bis(2-chloroethyl)-1-nitroso-
Bleomycin: NSC-125066
Cyclophosphamide: NSC-26271; CAS reg. No. 6055-19-2; 2*H*-1,3,2-oxazaphosphorine, 2-[bis(2-chloroethyl)-amino]tetrahydro-, 2-oxide, monohydrate
5-Fluorouracil: NSC-19893; CAS reg. No. 51-21-8; 5-FU
Methotrexate: NSC-740; CAS reg. No. 59-05-2; glutamic acid, *N*-[*p*-[[(2,4-diamino-6-pteridinyl)methyl]-methylamino]benzoyl]-

MOPP: combination of methotrexate, vincristine (Oncovin), prednisone, and procarbazine

NSC-45388: CAS reg. No. 4342-03-4; 5-(3,3-dimethyl-1-triazeno)imidazole-4-carboxamide

Prednisone: NSC-10023; CAS reg. No. 53-03-2; pregna-1,4-diene-3,11,20-trione, 17,21-dihydroxy-

Procarbazine: NSC-77213; CAS reg. No. 491-88-3; *p*-toluamide, *N*-isopropyl-α-(2-methylhydrazino)-, monohydrochloride

Vincristine: NSC-67574; CAS reg. No. 2068-78-2; leurocristine, sulfate (1:1)

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Some Determinants of the Therapeutic Efficacy of Actinomycin D (NSC-3053), Adriamycin (NSC-123127), and Daunorubicin (NSC-83142) [1,2]

Herbert S. Schwartz [3]

SUMMARY

Actinomycin D, adriamycin, and daunorubicin commonly interact with DNA but are also distinguished by diverse antineoplastic actions in human and animal tumors. Their antitumor efficacy is dependent upon selective toxicity, and our attention has been focused upon this toxicity. The selectivity of actinomycin D is correlated with drug retention at toxic concentrations for periods of time sufficient to produce histopathologic changes in sensitive normal tissues and in the Ridgway osteogenic sarcoma. The mechanisms responsible for its selective retention by sensitive tissues are not known, although it is clear that inhibition of RNA synthesis does not determine cytotoxicity. In a study of the actions of anthracyclines in P288 lymphocytic leukemia in mice, adriamycin has considerably greater antitumor activity than daunorubicin. The therapeutic advantage is lost, however, when the host is pretreated with immunosuppressive doses of whole body irradiation (250 R). This result suggests that daunorubicin may be more immunosuppressive than adriamycin. It is also consistent with observations that considerably higher concentrations of daunorubicin than adriamycin accumulate in the spleen of the mouse, whereas liver, thymus, and tumor cells exhibit no such difference in accumulation. Cytotoxicity may be related to the observation that susceptible cells exposed to daunorubicin at therapeutic doses show evidence of DNA damage.

[Cancer Chemother Rep 58:55–62, 1974]

In studies of anticancer agents, biochemical mechanisms of action may have only limited value in contributing to our understanding of selective toxicity. Actinomycin D, for example, binds to DNA and inhibits RNA synthesis. Nevertheless, this agent is selective in that actively proliferating cells are generally more sensitive to its toxic actions than those which are either not proliferating or are only minimally proliferating (1). Further evidence of selectivity is shown in that two human neoplasias are optimal therapeutic candidates for treatment with this agent: Wilms' tumor and choriocarcinoma.

There appear to be a number of factors which together cause a selective effect against some proliferative tissues. The uptake of actinomycin D by some cells may proceed until

[1] Supported in part by Public Health Service research grant CA-12533 and Core Program grant CA-13038 from the National Cancer Institute, National Institutes of Health, Department of Health, Education, and Welfare.

[2] Daunorubicin: CAS reg. No. 23541-50-6; daunomycin (NSC-82151).

[3] Department of Experimental Therapeutics and J. T. Grace Jr. Cancer Drug Center, New York State Department of Health, Roswell Park Memorial Institute, Buffalo.

levels of the drug exceed those necessary to inhibit RNA synthesis (1). At about ten times this minimal level, DNA replication is also blocked (2), and in such cells cytotoxicity may occur. Whether or not the cells are lethally affected, however, depends upon the duration of the blockade and upon the ability of the cells to sustain this blockade. For example, cytotoxicity in the bone marrow and intestinal epithelium may be incurred within 3 hours, in the spleen within 8–24 hours, and in the thymus within 24–48 hours.

The present report describes some past studies of actinomycin D which demonstrate selective drug retention by normal mouse tissues and by a sensitive experimental murine tumor. Recent studies demonstrate a different type of specificity with two other agents that also bind to DNA (3). These drugs, daunorubicin and adriamycin, have differential actions on host responses to immunizing tumors, and these effects will be described briefly.

Figure 1 shows the results of a survival study of AKR mice implanted with the Ridgway osteogenic sarcoma (ROS) and treated intravenously (iv) with single doses of actinomycin D (4). The tumors were allowed to grow to a palpable and measurable size before the drug was administered. The control animals (which received saline and mannitol) died at about 20 ± 10 days. With increasing doses of actinomycin D, there was a significant increase in the number of survivors that lived more than 100 days and showed no cytopathologic evidence of tumor at the site of implantation. At 800 μg/kg, 15 of 16 animals survived without evidence of tumor; at higher doses, early deaths due to toxicity were apparent.

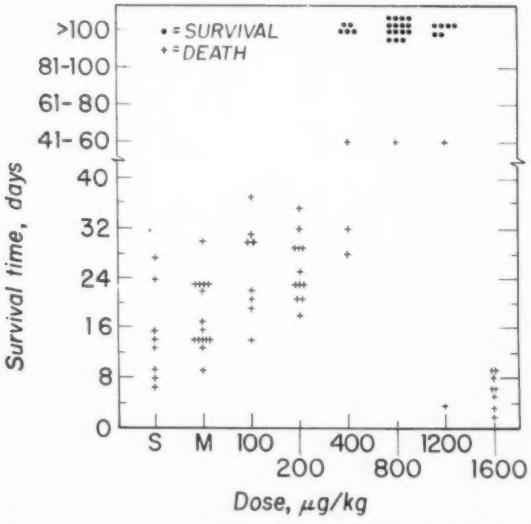

FIGURE 1.—Survival time of AKR mice bearing ROS after a single iv injection of actinomycin D. Control mice received saline or mannitol on Day 0 when the tumors had average diameters of 15–20 mm. [From Schwartz et al (4). Reprinted with permission from *Cancer Research.*]

The growth kinetics of ROS are shown in figure 2. When the tumor was approximately 10 mm in size (average diameter), the mice received single iv injections of actinomycin D (Day 0) (4). Tumors in the controls had relatively linear growth, whereas tumors in the

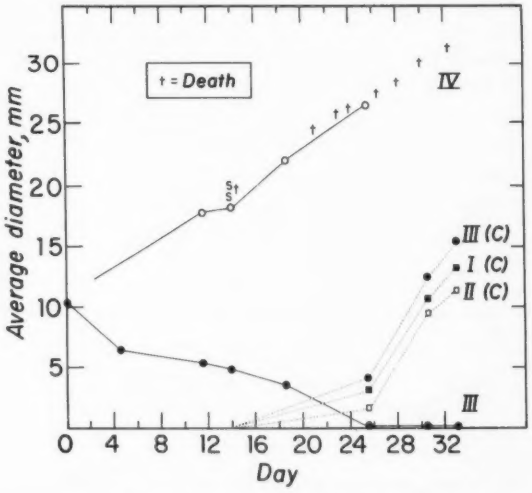

FIGURE 2.—Effects of a single dose of actinomycin D on ROS implanted before and after treatment. Average diameters of tumors implanted before treatment with 800 μg actinomycin D/kg (III) and saline (IV) are indicated by solid lines. Those of the challenge tumors (C) implanted 14 days after treatment are shown by dotted lines. IC and IIC did not receive the initial transplant but were treated with actinomycin D (IC) or saline (IIC) on Day 0. IIIC indicates the growth of challenge tumors in mice of group III. Mice of group IVC (8) (not shown) were all dead by Day 33 and three mice lived long enough to demonstrate growth of the challenge tumor. [From Schwartz et al (4). Reprinted with permission from *Cancer Research.*]

mice receiving only a single dose of drug regressed completely. Fourteen days after administration of actinomycin D, hosts were challenged with a second implantation of ROS. Up to approximately Day 26, this second tumor grew concurrently in the same mice in which the initial implant was regressing. In other studies (5), a tumor which was originally derived from 7,12-dimethylbenz(α)anthracene (DMBA) treatment was selected because it could grow concurrently in animals that were also carrying ROS. This DMBA-derived tumor was resistant to actinomycin D at maximally tolerated doses (800 μg/kg) given once iv. In mice bearing both ROS and DMBA tumor, the rate of growth of the DMBA tumor was unaltered even while the ROS regressed after this treatment. There was a question as to whether or not actinomycin D binds equally well to DNA of the two tumors. To test the binding of the drug to DNA (fig 3), isolated nuclei from ROS were incubated with actinomycin D and then lysed. The DNA was then centrifuged through a cesium chloride gradient and it was shown that actinomycin D was tightly bound to DNA in the concentrated salt solution during the course of centrifugation. In subsequent experiments, the binding of actinomycin D was compared in DNA from the ROS and DMBA tumor. It is apparent from results shown in figure 4 that the DNA from the resistant DMBA tumor binds actinomycin D at least as well as the DNA from the sensitive ROS.

An attempt has also been made to correlate the initial uptake of ^{3}H-actinomycin D by an array of tissues with the potential for the agent to produce cytotoxicity (1). The tissue concentrations at 30 minutes, for example, in liver, spleen, and salivary tissues, are uniformly high although each has a different cytotoxic response to actinomycin D (fig 5). The liver is relatively resistant to the cytopathologic effects induced by actinomycin D. The spleen, on the other hand, is a tissue which is sensitive to cytotoxicity from actinomycin D, and the salivary gland is a tissue of intermediate sensitivity. The salivary gland shows cytotoxicity only after a single maximally tolerated dose has been administered to the mouse.

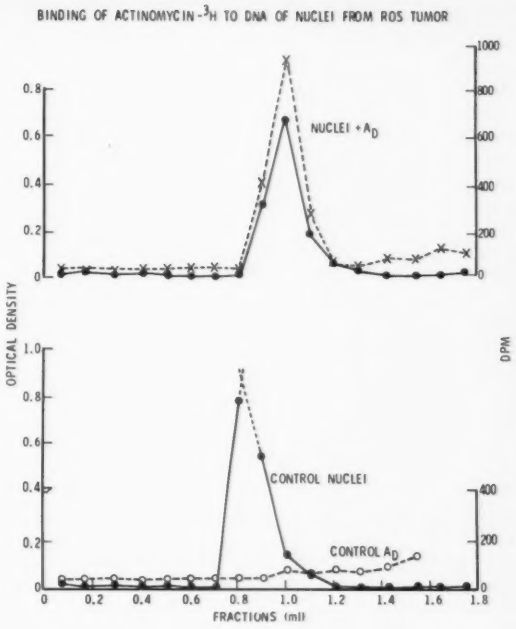

FIGURE 3.—Binding of ^{3}H-actinomycin D (A_D) to DNA of nuclei from ROS. Nuclei were isolated in TKM-sucrose (0.05 M tris buffer pH 7.4, 0.025 M KCl, 0.005 M $MgCl_2$, 0.25 M sucrose) and were incubated with ^{3}H-actinomycin D for 15 minutes at 37 C (*upper figure*). Control incubations (*lower figure*) were carried out without nuclei or without actinomycin D. Solutions were dialyzed at 4 C for 2 hours against 100 volume TKM-sucrose. The dialysates were treated with 0.025% Na lauryl sulfate and CsCl was added to give a final density of 1.72. Precipitates were removed by a brief centrifugation and the supernatants were then centrifuged at 47,000 rpm for 19 hours at 23 C in a Spinco SW 50.1 rotor. Optical density (●——●) and radioactivity (×——×, ○——○) were determined in 0.1-ml fractions drawn from the bottom of the tubes. All glassware was siliconized and nitrocellulose tubes were saturated with unlabeled actinomycin D prior to use.

During the early phase of drug uptake, ROS, thymus, and DMBA tumor all have relatively low uptake (1). Again, there is no correlation in this uptake between the amounts of the drug found and the relative fate of the cells in these tissues. For example, ROS is highly sensitive to the drug, the thymus is somewhat less sensitive, and the DMBA tumor is rela-

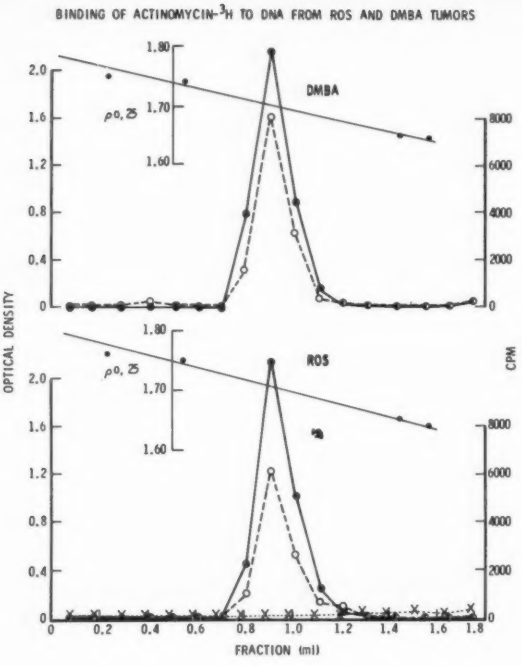

FIGURE 4.—Binding of ^{3}H-actinomycin to DNA from ROS and DMBA-induced tumors. NaDNA from tumor tissue was precipitated several times from alcoholic solutions after isolation in 0.15 M NaCl-0.01 M Na citrate. Solutions were incubated and centrifuged as described in figure 3.

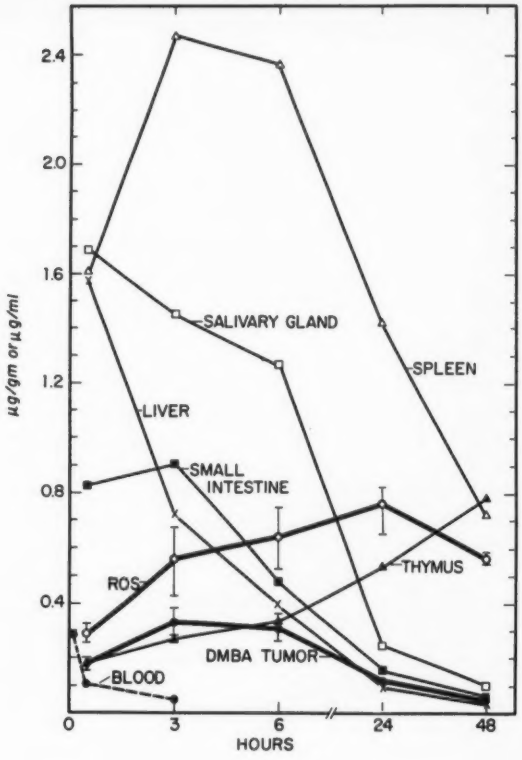

FIGURE 5.—Blood and tissue concentrations of drug in AKR mice at various times after administration of ^{3}H-actinomycin D.[From Schwartz et al (1). Reprinted with permission from *Cancer Research*.]

tively resistant. It is for such reasons that tissue toxicity was correlated with retention rather than with initial uptake. In this respect, studies of the inhibition of uridine incorporation into RNA by actinomycin D correlated well with drug levels at 3 hours but not with cytotoxicity in the various tissues. This indicates that inhibition of RNA synthesis is not necessarily a determinant of future cytotoxicity. Inhibition of RNA synthesis at 3 hours can be shown in tissues that contain large amounts of actinomycin D: spleen, liver, salivary gland, and intestine. ROS, in contrast, shows essentially no early inhibition, and it is only much later that incorporation is blocked. Spleen, thymus, and ROS, the most sensitive tissues, have accumulations of actinomycin D which are approximately 1 μg/g of tissue by 48 hours—concentrations which produce cytotoxicity. Other tissues (except for that of the small intestine) have considerably less retention and show little or no cytotoxicity.

The small intestine is different from the other organs however, in that it maintains relatively high concentrations of the drug for only 3–6 hours and yet it is most severely damaged by the drug. The drug levels are very low at 24 hours and are even lower still at 48 hours. The sensitivity of intestinal epithelium to actinomycin D is demonstrated 2–3 hours after a single iv dose, when cells in lower portions of the crypts begin to develop the typical pathologic changes that are associated with inhibition of mitosis and subsequent cell death. Karyorrhexis and loss of polarity pro-

ceed, becoming increasingly severe until about 24 hours after the treatment when the tissue may show evidence of recovery.

This study helped, in some degree, to understand the toxic effects of actinomycin D on the sensitive and resistant tissues. The liver, for example, is highly resistant and loses its drug rapidly. The salivary gland is moderately resistant but holds its drug. The small intestine is highly sensitive, and although the drug is lost rapidly, the onset of cytotoxic changes appear even before the drug levels fall to ineffective concentrations.

Daunorubicin and adriamycin, like actinomycin D, also bind to DNA. There are differences in their biochemical and pharmacokinetic activities, but the striking effect of these two antibiotics is that they are each highly selective against different tumors despite their remarkably similar structures. We selected P288 lymphocytic leukemia, which shows a differential antitumor response to adriamycin and to daunorubicin (3). The drugs were administered intraperitoneally (ip) daily for 5 days, and the average survival time (AST) was determined (fig 6). Untreated mice have an AST of about 12 days. Mice bearing P288 have a minimal response to daunorubicin, but demonstrate a striking increase in survival time after adriamycin is administered, with long-term survivors (> 50 days) ranging from about 10% to 30% of the total number receiving therapeutic doses. In subsequent studies, a single-dose regimen was administered to the animals bearing the same tumor and, again, adriamycin was consistently more effective than daunorubicin both in the prolongation of survival and in the number of long-term survivors (fig 7).

To eliminate the possible participation of an immunologic response by the host, mice were given a low dose of ionizing radiation (250 R) on the day before the tumors were implanted (Day 0). Animals were treated as before on the day after implantation (Day 1); the results are shown in figure 8. In irradiated animals, there was no difference in ASTs of mice treated with adriamycin or daunorubicin at any dose level (3). In contrast, the sham-irradiated mice showed increased survival times with approximately four of 20 animals becoming long-term survivors after adriamycin therapy.

We also tested the effects of concurrent therapy using adriamycin and methotrexate [4]

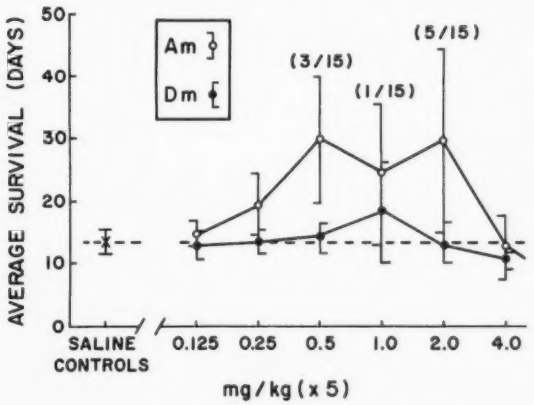

FIGURE 6.—Average survival of DBA/2J mice bearing P288 ascites and treated once daily for 5 days ip with adriamycin (Am) or daunorubicin (Dm). In parentheses are the number of 50-day survivors over the total number treated. Implantation was on Day 0 and treatment commenced on Day 1. [From Schwartz et al (3). Reprinted with permission from *Cancer Research*.]

[4] Methotrexate: NSC-740; CAS reg. No. 59-05-2; glutamic acid, *N*-[*p*-[[(2,4-diamino-6-pteridinyl)methyl]methylamino]benzoyl]-.

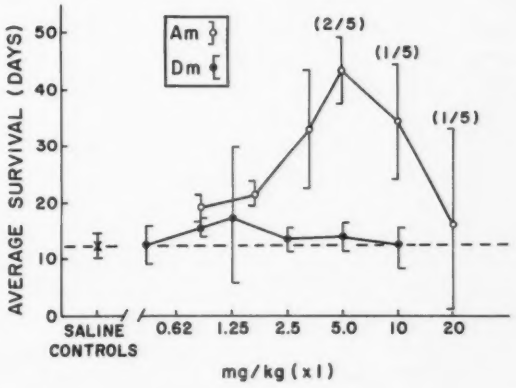

FIGURE 7.—Average survival of mice bearing P288 and treated once ip with adriamycin (Am) or daunorubicin (Dm) on Day 1. Conditions are as described in figure 6. [From Schwartz et al (3). Reprinted with permission from *Cancer Research*.]

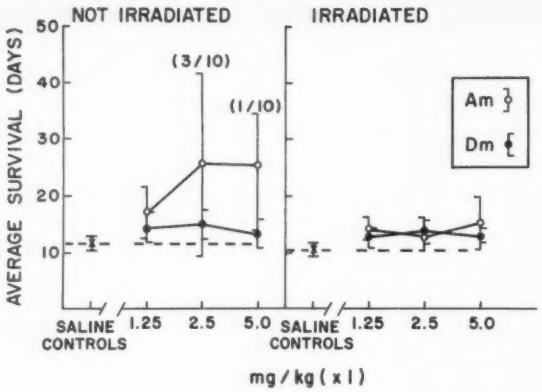

FIGURE 8.—Average survival of irradiated and sham-irradiated mice bearing P288 and treated once ip with adriamycin (Am) and daunorubicin (Dm). Mice were given 250 R whole body irradiation or were sham-treated on the day prior to implantation of the tumor (Day 0). Drugs were administered on Day 1 as described in figure 7.

in mice bearing the P288 tumor. Methotrexate, which is well known as an immunosuppressive agent, also decreased the effectiveness of adriamycin (3). In mice bearing L1210 leukemia methotrexate was administered with adriamycin, and once more, it diminished the antitumor effects of the drug (3). The L1210 tumor was selected because it is even less immunizing in DBA/2J mice than the P288 leukemia. Yet, even as L1210 is less immunizing, so also is adriamycin less effective, giving essentially no more than a 75% increase in survival time with no long-term survivors. Mice were also pretreated with immunosuppressive doses of cyclophosphamide[5] (150 mg/kg) on the day before P288 implantation. The results obtained in these animals showed, like those described for pre-irradiated mice, that the efficacy of adriamycin was much reduced as compared to that of daunorubicin.

Studies of the uptake of adriamycin and daunorubicin were carried out with the P288 and L1210 tumors. There were essentially no differences in the uptake at various times after a single low dose of either drug, except during the first hour when uptake of adriamycin was delayed. Daunorubicin was taken up more rapidly and was maintained at slightly higher levels than adriamycin (3).

In a study of host tissues, there were also no differences in the amounts of drugs taken up by the thymus or the liver of normal mice over a 4-hour period (fig 9). But in the spleen, adriamycin was taken up at approximately one half the rate of daunorubicin (3,6). This appears to be consistent with a greater immunosuppressive effect by daunorubicin and suggests that adriamycin is perhaps a more effective agent because it is not immunosuppressive to the extent or in the manner of daunorubicin.

The concept of selectivity implies that there is a difference in toxic response by a tissue because of a property that is affected differently by a given drug. The problem is to find that differing effect which explains the specific response. For example, in these studies with the anthracyclines, the differential response of P288 cells to adriamycin and daunorubicin appeared to be tumor specific, but only until the tests were conducted in immunosuppressed mice. It was then apparent that the difference in response was due to a selective toxicity in the host rather than the tumor. In addition, uptake studies in the P288

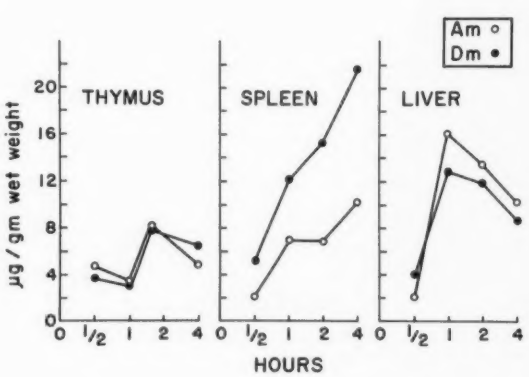

FIGURE 9.—Uptake of adriamycin (Am) and daunorubicin (Dm) (5 mg/kg iv) by tissues of mice without tumors. [From Schwartz et al (3). Reprinted with permission from *Cancer Research.*]

[5] Cyclophosphamide: NSC-26271; CAS reg. No. 6055-19-2; 2*H*-1,3,2-oxazaphosphorine, 2-[bis(2-chloroethyl)-amino]tetrahydro-, 2-oxide, monohydrate.

tumor, the liver, and the thymus showed no differential effect, but the results with the spleen did. This specific difference in a lymphoid organ was consistent with the relative toxicities of the drugs in bone marrow (7) and with the results obtained in immunosuppressed mice. For these reasons, the differences between the agents seem clearly related to a differential toxicity to some lymphoid cells.

Responses by the spleen and bone marrow to the anthracyclines appear to be well correlated with some of their differential tumor actions. In pursuing the basis for differential cytotoxicity, however, more detailed investigations may provide some insight into mechanisms that contribute to their selectivity. In spleen colony assays, Razek et al (7) demonstrated that daunorubicin is more cytotoxic to marrow stem cells than is adriamycin. Our current studies, confirming this observation, also indicate that adriamycin may be the more effective drug in blocking the differentiation of these stem cells. In this regard, actinomycin D selectively inhibits 19S antibody production, an effect which is in contrast to the more general inhibition of 7S antibody production by a variety of other agents (8).

Another observation suggests that a mechanism of anthracycline action may involve damage to DNA. In preliminary experiments, mice bearing P288 were pretreated for 18–24 hours with ^{14}C-thymidine to label the DNA. Animals then received saline or daunorubicin (5 mg/kg ip) and were killed up to 3 hours later. The results, shown in figure 10, indicate that the DNA of cells from treated animals is extensively nicked. Although these experiments have not yet been extended to include adriamycin or studies with normal tissues, the results are consistent with the recent report that both anthracyclines as well as actinomycin D inhibit mammalian repair processes (9).

How might such a drug action be selective? It seems reasonable to speculate that toxicity may in fact be due to the actions of the drugs themselves or their metabolites on the DNA or on its repair mechanisms. It may be important, for example, to know whether myocardial lesions are related to such actions. This

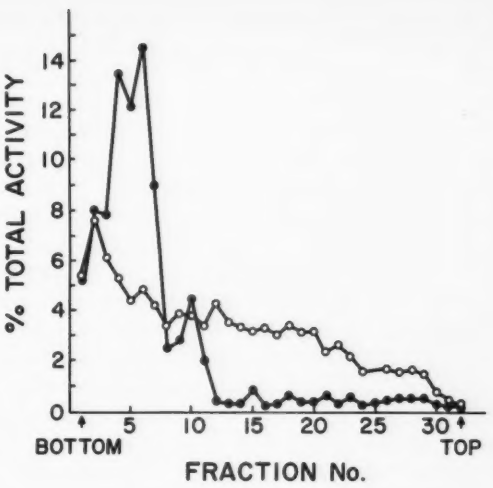

FIGURE 10.—Alkaline sucrose (5%–20%) gradients of DNA from P288. Conditions are described in the text. Daunorubicin-treated animals (○——○); saline controls (●——●).

chronic cardiac toxicity, which is limiting in patients receiving high doses of either anthracycline, appears to involve a slow deterioration of myocardial tissues over a period of weeks and months after treatment. This might be due to an inability of the human tissue to repair damaged DNA by prior exposure to these agents. It appears to be a species-specific lesion, since mice, rats, and dogs do not demonstrate such chronic toxicity. Further, if cardiac toxicity is due to an aglycone metabolite, it might be possible to prevent the pathologic changes by inhibition of the hydrolyzing enzymes. This system involves the microsomal mixed function oxidases, and inhibition by irradiation or chemotherapy, prior to administration of adriamycin or daunorubicin, decreases formation of the aglycones (3,9).

These speculations serve to direct our attention toward mechanisms of selectivity. It seems equally important, in retrospect, to consider once more the mechanisms that are responsible for determining selectivity to actinomycin D. The concept of DNA binding is not adequate per se to explain differences in retention by various tissues or tumors. Binding and retention of actinomycin D and the an-

thracyclines may be affected by changes during the mitotic cycle of proliferating cells in some manner which is not clear but which may determine selectivity. Binding may be determined wholly or in part by the conformation of exposed DNA during G_1 or S phases (10), or by the nuclear histones that may envelop DNA selectively during different phases of the cell cycle. Secondary drug binding and intracellular redistributions in various tissues are also possibilities that have not yet been critically investigated.

Further, it seems reasonable to expect that analogs of this class of drugs which bind to DNA will have different patterns of selectivity. Until we are able to describe the factors that determine selectivity, whether metabolic or intrinsic in other ways, to a given cell population, the search for more useful agents and their clinical applications remains essentially empirical. Yet it is abundantly clear that this class of agents which interact directly with DNA continues to offer unexpected selective advantages against a variety of human neoplasias.

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Evaluation of Actinomycins in Experimental Systems

Abraham Goldin and Randall K. Johnson [1]

SUMMARY

The antitumor activity of actinomycin D has been evaluated in a number of experimental systems. It is highly active against Ridgway osteogenic sarcoma, P388 leukemia, B16 melanotic melanoma, and adenocarcinoma 755. Actinomycin D has definite antitumor activity in several other experimental solid and hematologic tumors including L1210 leukemia. Extensive testing in L1210 leukemia has shown that the therapeutic effect of actinomycin D is not dependent on schedule of administration. The toxicity of this antibiotic in normal mice is cumulative and host recovery is slow. No schedule of administration offers any apparent advantage in decreased host toxicity. In combination chemotherapy trials in L1210 leukemia, actinomycin D appeared to be synergistic with methotrexate and emetine but not with vincristine or L-asparaginase. However, marked therapeutic potentiation was observed for actinomycin D plus L-asparaginase in L5178Y leukemia. Several naturally occurring or synthetic actinomycin derivatives have been screened. To date, none of these has shown an advantage over actinomycin D which would warrant development toward clinical trial.

[Cancer Chemother Rep 58:63–77, 1974]

Recent developments in cancer chemotherapy, particularly the clear-cut superiority of adriamycin as opposed to daunorubicin in a variety of human tumors (1), have prompted a renewed look at the clinical and experimental antitumor effects of antibiotics such as actinomycin D. It is possible that intensive reinvestigation of the biologic properties of such compounds could lead to improved methods of therapy or the development of analogs with greater therapeutic effectiveness.

The quantitative evaluation of antitumor activity of drugs in vivo must take into account not only the effect of the drug on the tumor but all of the complex interrelationships between drug, host, and tumor shown in figure 1 (2). The therapeutic endpoint used, whether it is inhibition of tumor growth or increase in host survival, is actually a balance of these diverse factors: the cytotoxic action of the drug on the tumor cells; the effects of the drug on the host (not only overt toxicity but also modification of the host's immune response to the tumor); the lethality of the tumor to the host; the effects of the tumor on the drug, such as the development of resistance, binding, or incorporation into tumor cell macromolecules; and the effects of the host on the drug, ie, distribution binding, metabolism, and excretion.

The assay system most extensively used in evaluating antitumor activity in the Drug Research and Development program of the National Cancer Institute is the L1210 lymphoid leukemia. An example of the therapeutic response of L1210 leukemia is shown in figure 2 (3). As the dose of an effective agent, in this

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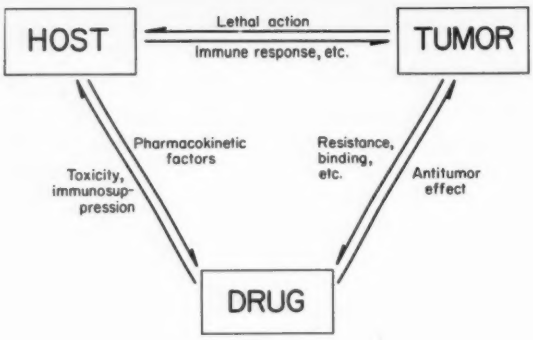


FIGURE 1.—Interrelationships between drug, tumor, and host which influence chemotherapeutic efficacy. Originally described by Marshall (2) in relation to the effectiveness of antimicrobial agents.

case methotrexate,[2] is increased, the survival time of tumor-bearing mice is increased. Survival time reaches a maximum at the optimal dose, and then, as the dose is further increased, it decreases as toxicity becomes dominant. The height of the curve is a measure of the effectiveness of the drug, and the width of the curve gives some indication of the degree of safety with which a drug can be employed. The narrower the curve, the smaller the therapeutic index for any given agent.

This type of data permits a quantitative comparison of antitumor effectiveness among various agents. Figure 3 (4) compares the activities in advanced L1210 leukemia of methotrexate, 6-thioguanine, 6-mercaptopurine, and azaserine, four agents with inhibitory effects of de novo purine biosynthesis (5–8). Methotrexate is about twice as effective in this tumor system as 6-thioguanine or 6-mercaptopurine. Azaserine is the least effective of the four agents. The dosage range of antitumor effectiveness of the two purine antimetabolites is quite dissimilar despite the fact that they are such closely related compounds. Also, there is the suggestion that 6-thioguanine, having a broad

[2] Chemical nomenclature, NSC numbers, and CAS registry numbers for all compounds mentioned in this paper are included in the List of Compounds at the end of the text.

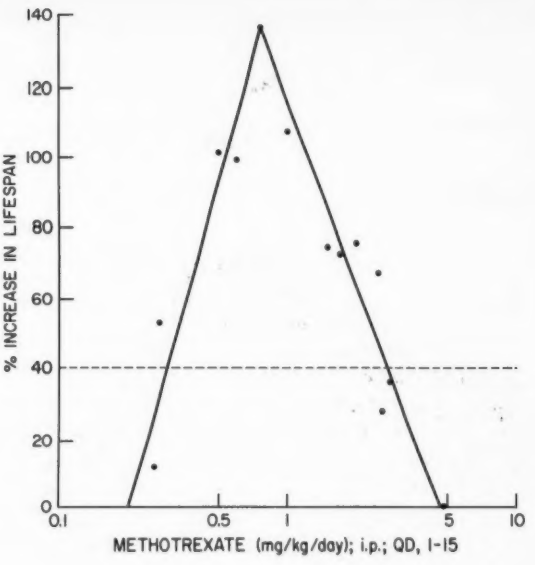


FIGURE 2.—Influence of daily methotrexate on the survival time of mice inoculated ip with 10^5 L1210 leukemia cells. Average data from 313 experiments are shown. Data of Skipper and Schmidt (3). Maximum effectiveness = ILS at optimal dose = 136%. Therapeutic index = $\frac{\text{optimal dose}}{\text{dose which gives 40\% ILS}} = \frac{0.76}{0.3} = 2.5$.

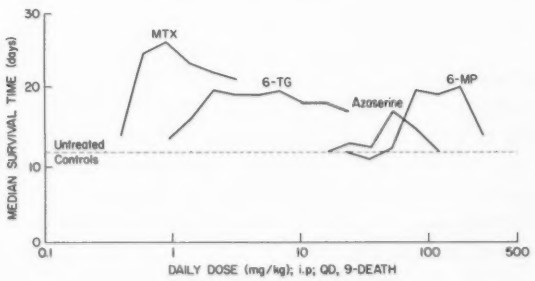


FIGURE 3.—Comparison of the effects of methotrexate (MTX), 6-thioguanine (6-TG), azaserine, and 6-mercaptopurine (6-MP) in the treatment of advanced sc L1210 leukemia (4). Optimal daily doses and maximum effectiveness in this experiment were: MTX, 0.9 mg/kg/day, 117% ILS; 6-TG, 2–7 mg/kg/day, 62% ILS; azaserine, 54 mg/kg/day, 42% ILS; 6-MP, 180 mg/kg/day, 67% ILS.

range of therapeutically effective doses, could be used with a wider margin of safety. This type of assay system can be used for studying congeners (9), for selecting optimal schedules of drug administration (10), and for studying the therapeutic efficacy of drugs used in combination (11).

An important adjunct to the determination of antitumor activity of a drug is the evaluation of its toxicity in non-tumored host animals. Dosage-mortality curves for actinomycin D in BDF_1 mice on several schedules of administration are shown in figure 4. Actinomycin D lethality rises sharply in the toxic dose range; on most schedules the LD90 is reached at about twice the LD10 dose. The LD10 dosage levels on the various schedules calculated from regression lines are listed in table 1. Comparison of

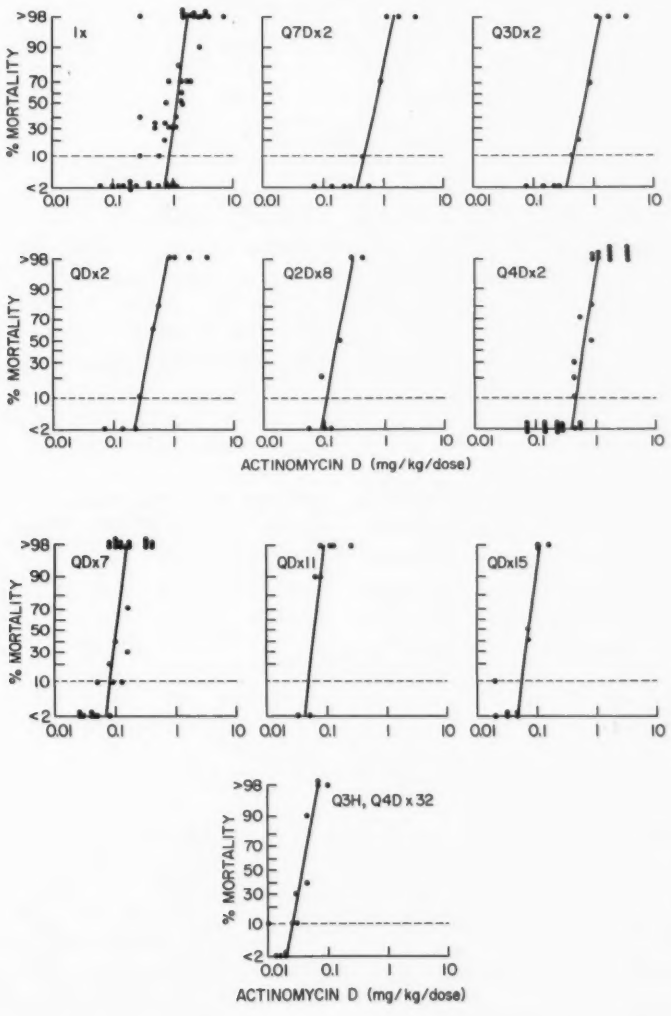


FIGURE 4.—Toxicity of actinomycin D on several schedules of administration in normal BDF_1 mice. Percent mortality is plotted in a probit transformation on the ordinate. The dose of actinomycin D in mg/kg/injection is plotted logarithmically on the abscissa. Each point represents a group of 6–10 mice treated at a given dosage level. Regression lines are drawn through the points. The dashed line represents the LD10 level.

TABLE 1.—Single-dose and total-dose LD10 levels for actinomycin D in BDF_1 mice

Schedule	LD10 (mg/kg)*		
	Per dose*	Total dose	Total dose relative to single dose
One dose	0.87	0.87	1.0
qd × 2	0.29	0.58	0.7
q3d × 2	0.47	0.94	1.1
q4d × 2	0.47	0.94	1.1
q7d × 2	0.45	0.90	1.0
qd × 7	0.080	0.56	0.6
qd × 11	0.050	0.55	0.6
qd × 15	0.054	0.81	0.9
q2d × 8	0.11	0.88	1.0
q3h × 8, q4d × 4	0.026	0.83	1.0

*Calculated from regression lines in fig 4.

the total-dose LD10 values can provide useful information about host recovery time from the toxic effects of an agent (12). The total-dose LD10 levels for actinomycin D indicate that the host toxicity of this agent is close to cumulative. It is also evident that host recovery from actinomycin D is slow, because the per-injection LD10 level for two doses at an interval of 7 days is well below the single-dose LD10.

The activity of actinomycin D in the L1210 leukemia assay system is summarized in figure 5. These results are compiled from several hundred experiments with actinomycin D treatment of early intraperitoneally (ip) implanted L1210 leukemia. It is apparent that the therapeutic efficacy of this drug in L1210 leukemia is not dependent on the schedule of administration. The optimal dose on any schedule results in a 40%–50% increase in survival time of tumor-bearing mice. The width of the dose-response curves suggests that administration of actinomycin D every third day may allow a broader range of effective doses (increase in lifespan [ILS] $\geq$ 25%), hence, a somewhat better margin of safety.

In a single experiment (data not shown) in which actinomycin D was administered daily by the oral route to mice bearing L1210 leukemia, no significant antileukemic effect or toxicity was seen at a dose (400 μg/kg/day) which is lethal on parenteral administration. This suggests that actinomycin D is absorbed poorly, if at all, from the gastrointestinal tract. Actinomycin D has the same order of activity in parenteral treatment of advanced subcutaneously (sc) implanted L1210 leukemia as it has against the early ip implanted tumor (table 2) (13).

The P388 leukemia assay system has been used extensively in the screening of fermentation and plant products, because it appears to be more sensitive to most natural products than is L1210 leukemia (14). This applies to actinomycin D as shown in table 3. Administra-

TABLE 2.—Effect of actinomycin D on lifespan of mice bearing advanced sc L1210 leukemia*

Compound	Schedule	Dosage range (mg/kg/dose)	Optimal dose (mg/kg)	MST† (days)	%ILS
Actinomycin D	qd, Day 7 to death	0.05–0.80	0.20	12.5	39
	q2d, Day 7 to death	0.10–1.6	0.20	14.0	56
	q4d, Day 7 to death	0.10–1.6	0.40	11.5	28
Methotrexate	qd, Day 7 to death	0.20–3.2	0.80	23.0	156
Controls	—	—	—	9.0	—

*Data from ref 13.
†MST = median survival time.

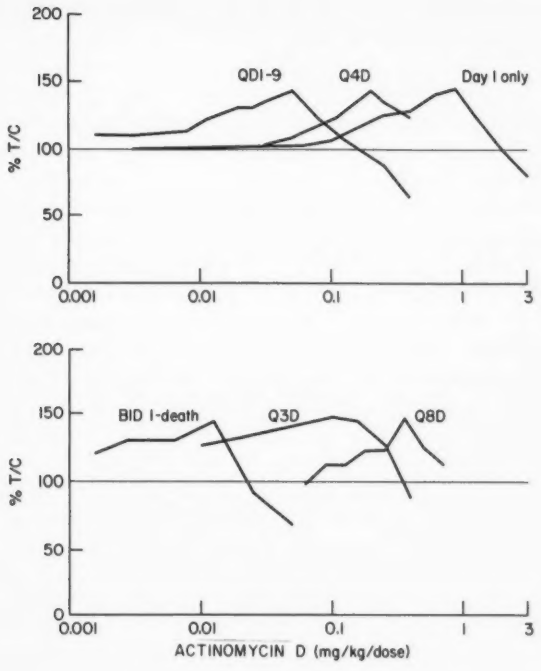


FIGURE 5.—Influence of treatment schedule on the therapeutic effectiveness of actinomycin D in mice bearing early ip L1210 leukemia. Maximum effectiveness on all schedules was approximately 45% ILS. Optimal doses on the various schedules were: qd, Days 1–9, 0.05 mg/kg/dose; q4d, Days 1,5,9, 0.2 mg/kg/dose; Day 1 only, 0.9 mg/kg/dose; twice daily, Day 1 to death, 0.012 mg/kg/dose; q3d, Days 1,4,7,10, 0.1 mg/kg/dose; q8d, Days 1 and 9, 0.35 mg/kg/dose.

tion of this antibiotic on daily or intermittent schedules markedly prolongs the lifespan of BDF_1 mice bearing ip implanted P388 leukemia and results in long-term survivors.

B16 melanotic melanoma is a slow-growing, low growth fraction tumor relative to the L1210 and P388 leukemias (15). The activity of actinomycin D in this assay system is shown in table 4. More than 50% ILS in mice bearing early ip implanted B16 melanoma is obtained after treatment with actinomycin D on several schedules. Prolonged daily treatment resulted in 100% ILS at the optimal dose. Advanced B16 melanoma, which is resistant to most chemotherapeutic agents, also responds to actinomycin D treatment (table 5).

TABLE 3.—Comparative activity of natural products in L1210 and P388 leukemias

Compound	Optimal %ILS*	
	L1210	P388
Actinomycin D	45	Cure†
Adriamycin	60	Cure†
Daunorubicin	50	130
Mitomycin C	100	150
Vincristine	38	140
Vinblastine	40	120
DON	60	90
Pactamycin	22	45
Mithramycin	10	90

*%ILS at optimal dose and schedule.
†Greater than 50% long-term survivors at optimal dose.

Lewis lung carcinoma, a slow-growing solid tumor which metastasizes to the lungs after sc or intramuscular (im) implantation, is resistant to treatment with many drugs which have a high degree of activity in other experimental tumor systems (16). Actinomycin D activity

TABLE 4.—Influence of schedule in actinomycin D treatment of early ip B16 melanoma*

Schedule	Dose (mg/kg/day)	%ILS
Once, Day 1	0.512	Toxic
	0.256	45
	0.128	30
qd, Days 1–9	0.100	Toxic
	0.050	71
	0.025	61
	0.012	55
qd, Days 1–17	0.064	Toxic
	0.032	100
	0.016	85
	0.008	40
q4d; Days 1,5,9	0.512	Toxic
	0.256	65
	0.128	50
	0.064	30
q4d; Days 1,5,9,13,17	0.256	Toxic
	0.128	63
	0.064	50
	0.032	20

*Actinomycin D administered ip. Median survival time of control groups was 16–20 days.

TABLE 5.—Effect of actinomycin D on lifespan of mice bearing advanced ip B16 melanoma*

Schedule	Dose (mg/kg/day)	%ILS
qd, Days 5–14	0.20	17
	0.10	103
	0.05	64
	0.025	39

*Actinomycin D administered ip. Median survival time of control animals was 14 days.

in this assay system is compared with several other fermentation products in table 6. Daily administration of this agent has a moderate effect on the growth of the primary tumor as indicated by the 57% tumor weight inhibition. However, this inhibition is not reflected in prolongation of lifespan of the tumor-bearing mice.

AK leukemia is a spontaneous, Gross virus-induced leukemia vertically transmitted in AKR mice (17). This spontaneous tumor has been used to study remission induction and maintenance (18), and single-drug and combination chemotherapy (19). Actinomycin D has some degree of activity in this system when given as a single dose on the day after diagnosis of the disease (table 7). Repetitive intermittent administration of this agent was toxic at all doses tested.

The tumor systems described above are those currently in extensive use in our program for the evaluation of antitumor agents. Actinomycin D has been tested in several other in vivo assay systems as well. Table 8 is a summary of actinomycin D activity against in vivo tumor systems. Actinomycin D treatment produces long-term survivors in only P388 leukemia and Ridgway osteogenic sarcoma (20). Of those tumor systems in which it has definite activity, it is most active against B16 melanoma, lymphoma 4, and adenocarcinoma 755. It had borderline activity in L5178Y lymphatic leukemia and Walker carcinosarcoma 256 which are sensitive to many of the clinically active antitumor agents. Actinomycin D was inactive in several other tumor systems, some of which (ie, Dunning leukemia, P1534 leukemia, and P1798 lymphosarcoma) are quite sensitive to a wide spectrum of drugs.

Cross-resistance studies with actinomycin D have been limited. An actinomycin D-resistant subline of a sensitive tumor has not been isolated and studied in our program. Sublines of L1210 and P288 leukemias resistant to methotrexate have retained sensitivity to actinomycin D. Chinese hamster cells in culture which have acquired resistance to actinomycin D proved to be cross-resistant to mithramycin, vincristine, vinblastine, daunorubicin, and mitomycin C (21). These cells retained sensitivity to methotrexate, 6-mercaptopurine, hydrocortisone, mechlorethamine, and several other agents. The mechanism of resistance proved to be an alteration of permeability. The degree of

TABLE 6.—Activity of fermentation products on tumor growth and survival time of mice bearing im Lewis lung carcinoma*

Compound	Tumor growth		Survival time	
	OD†	%TWI‡	OD†	%ILS
Bleomycin	18	96	18	58
Mitomycin C	2	25	1	42
Chromomycin A_3	0.2	20	0.02	25
Neocarzinostatin	0.1	42	0.06	24
Streptozotocin	100	39	200	21
Adriamycin	1	55	1	15
Mithramycin	0.125	50	0.125	14
DON	0.03	25	0.007	14
Actinomycin D	0.01	57	0.01	10

*Data of Microbiological Associates, Inc., Bethesda, Md.
†OD = optimal dose (mg/kg/day). Treatment on Days 1–11.
‡%TWI = percent tumor weight inhibition as measured on Day 12.

TABLE 7.—Response of spontaneous AK leukemia to some clinically active antitumor agents*

Compound	Optimal dose [(mg/kg)/schedule]	%ILS	%Cure
Cyclophosphamide	200/q10d × 4	164	13
	150/q7d × 7	238	18
Vincristine	0.1/q7d × 5	66	17
	0.25/Days 1,5,15,19	79	0
Adriamycin	2.1/qd × 6	47	0
Vinblastine	0.5/q8d × 2	40	0
Daunorubicin	3.5/qd × 6	36	0
Actinomycin D	0.1/Day 1 only	47	0
	0.14–0.41/q4d to death	0 (toxic)	
Bleomycin	6.7/qd × 10	5	6

*Frei E, Schabel FM, Jr, and Goldin A. Unpublished data.

cross-resistance to the above drugs was a function of their molecular weight. A subline of the same cultured cell line resistant to daunorubicin was cross-resistant to actinomycin D (22).

A comparison of activity in a spectrum of tumor systems between actinomycin D and six of the most active antitumor agents in the clinic is shown in table 9. Actinomycin D is among the least active of these clinically effective drugs in eight of the 11 experimental tumor systems.

TABLE 8.—Activity of actinomycin D in experimental tumor systems*

Tumors which could be cured by actinomycin D treatment	Tumor systems in which actinomycin D markedly increased lifespan or inhibited local tumor growth	Tumors against which actinomycin D had borderline activity	Tumor systems in which actinomycin D was inactive
P388 leukemia	B16 melanotic melanoma (100)	L5178Y lymphatic leukemia (26)	ADJ-PC-20 plasma cell
Ridgway osteogenic sarcoma	L1210 leukemia (45)	P355 leukemia (30)	ADJ-PC-22 plasma cell
	Gardner 6C3HED lymphosarcoma (75)	Lymphoma 8 (rat) (26)	Lieberman plasma cell No. 1 (LPC-1)
	Mecca lymphosarcoma (83)	Murphy-Sturm lymphosarcoma (rat) (78% TWI)	Friend virus leukemia
	P288 lymphocytic leukemia (55)	Walker carcinosarcoma 256 (rat) (65% TWI)	Hepatoma 129
	P1081 chloroleukemia (145)	AK leukemia (0–47, variable)	AK4 lymphoid leukemia
	P329 reticulum cell sarcoma (66)		Lewis lung carcinoma
	Lymphoma 4 (94)		MPC2 plasma cell
	Sarcoma 180 (83% TWI)		Osteogenic sarcoma He 10734
	Adenocarcinoma 755 (100% TWI)		P1534 leukemia
			P1798 lymphosarcoma
			Dunning leukemia (rat)
			Hamster tumors
			Adenocarcinomas of the duodenum, pancreas, prostate, kidney, and small bowel
			Plasmacytoma No. 1
			Melanotic melanoma
			Cystadenocarcinoma of the liver No. 1

*Numbers in parentheses are optimal %ILS or tumor weight inhibition (TWI).

It is among the most highly active against three of the tumors: P388 leukemia, B16 melanoma, and adenocarcinoma 755.

There have been relatively few evaluations of the efficacy of actinomycin D in combination with other agents in experimental tumor systems. Combinations of actinomycin D plus methotrexate have been evaluated in the advanced L1210 leukemia system (table 10). A sequential schedule of drug administration, in which a single dose of actinomycin D was followed by daily methotrexate, did not result in a therapeutic response better than that obtained with methotrexate alone. However, on a somewhat different schedule of weekly actinomycin D administration plus daily treatment with methotrexate, data from two experiments suggest that the combination is more effective than either agent alone in the treatment of advanced sc implanted L1210 leukemia.

The combination of actinomycin D and vincristine, two agents effective individually in the treatment of Wilms' tumor, choriocarcinoma, and testicular tumors (23), has been tested in the ip implanted L1210 leukemia system. Sequential administration of these drugs on Days 1 and 2 after tumor implantation resulted in a moderate ILS which was not clearly superior to ILSs from treatment with either agent alone (table 11). These data are from a single experiment and it is possible that the administration of these two agents on a different schedule could result in therapeutic synergism.

The combination of actinomycin D plus emetine, an inhibitor of protein biosynthesis in eukaryotic cells (24) which has antitumor activity in L1210 leukemia (25), proved to be therapeutically synergistic in early ip implanted L1210 leukemia (table 12). On two schedules the combination yielded greater ILSs than the single agents: administration of actinomycin D daily plus emetine every fourth day and administration of both drugs every fourth day. In the latter experiment the interval between administration of the two drugs was varied from 24 hours to simultaneous injection. At all of the 13 intervals studied the median survival times of the groups receiving the combination were greater than those of the groups treated with either drug alone. The maximum ILS of 79% was observed when a dose of actinomycin D was followed in 6 hours by a dose of emetine.

Combination chemotherapy has also been studied with actinomycin D plus L-asparaginase, another agent with the primary mode of action of protein synthesis inhibition (in cells which require exogenous L-asparagine) (26). L1210 leukemia cells have the enzyme, L-asparagine synthetase, and are thus resistant to the cytotoxic effects of L-asparaginase (27). It is not surprising then, that the combination of actinomycin D plus L-asparaginase is no more effective in the treatment of mice bearing L1210 leukemia than actinomycin D alone (table 13). This same two-drug combination has been tested in L5178Y leukemia which is L-asparaginase sensitive (fig 6) (28). In this experiment the single agents were only moderately active, while the combination was markedly synergistic, resulting in long-term survivors.

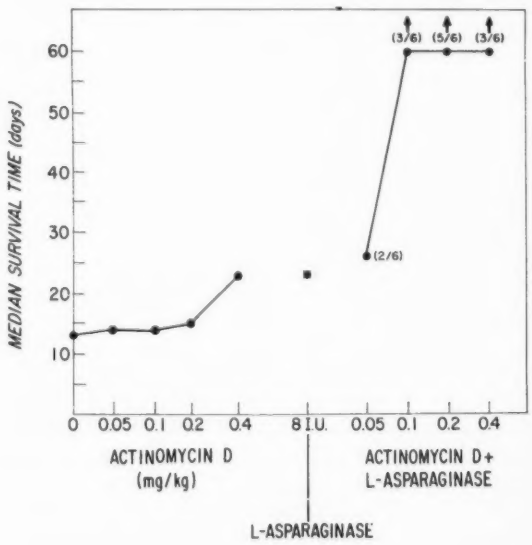


FIGURE 6.—The effects of actinomycin D alone and in combination with L-asparaginase on the survival of mice bearing L5178Y lymphatic leukemia. L-Asparaginase was given ip daily from Days 1 to 5 at a dose of 8 IU/mouse. Actinomycin D was given at the doses shown on Days 1 and 5. Median survival time of untreated control mice was 13 days. Numbers in parentheses are 60-day survivors over number of mice treated. Data of Jacobs et al (28).

TABLE 9.—Optimal ILS or tumor weight inhibition (TWI) of seven clinically active antitumor agents

	%ILS									% TWI	
Drug	L1210 leukemia	P388 leukemia	B16 melanoma	Lewis lung carcinoma	P1534 leukemia	P288 leukemia	L5178Y leukemia	Dunning leukemia	Gardner lympho-sarcoma	Walker 256 carcino-sarcoma	Adenocar-cinoma 755
Actinomycin D	45	Cure†	100	10	0	55	26	20	75	65	100
Methotrexate	140	120	0	15	60	190	88	95	200	95	55
Cyclophosphamide	160	200	60	Cure	70	100	•	Cure	200	90	100
5-Fluorouracil	95	120	45	25	•	90	55	65	>120	72	95
BCNU	Cure	Cure	110	45	165	100	160	Cure	200	100	85
Vincristine	40	140	80	0	Cure	80	25	80	200	80	45
Adriamycin	50	Cure	Cure	15	•	•	•	•	•	90	•

*Not tested.
†Greater than 50% long-term survivors after optimal treatment.

TABLE 10.—Combination chemotherapy of advanced sc L1210 leukemia with actinomycin D plus methotrexate*

Compound	Schedule	Optimal dose (mg/kg)	%ILS
Actinomycin D	Day 6 only	0.65	30
Methotrexate	qd, Day 7 to death	0.39	130
Actinomycin D + Methotrexate	Day 6 only + qd, Day 7 to death	0.23 + 0.39	135

		Expt 1	*Expt 2*	*Expt 1*	*Expt 2*
Actinomycin D	q7d, Day 5 to death	1.08	0.65	37	37
Methotrexate	qd, Day 6 to death	0.65	1.80	137	81
Actinomycin D + Methotrexate	q7d, Day 5 to death + qd, Day 6 to death	0.23 + 0.65	0.23 + 0.65	238	112

*Data of Microbiological Associates, Inc., Bethesda, Md. 10^5 L1210 cells implanted sc, sc treatment.

TABLE 11.—Sequential combination treatment of early ip L1210 leukemia with actinomycin D plus vincristine*

Compound	Schedule	Optimal dose (mg/kg)	%ILS
Actinomycin D	Day 1	0.8	22
Vincristine	Day 1	4.0	33
Actinomycin D	Days 1 and 2	0.2	33
Vincristine	Days 1 and 2	1.0	33
Actinomycin D + Vincristine	Day 1 + Day 2	0.4, 0.2, 0.2 + + + 4.0, 4.0, 1.0	39, 44, 39
Actinomycin D + Vincristine	Day 2 + Day 1	0.2, 0.1, 0.2 + + + 2.0, 2.0, 1.0	33, 44, 33

*Data of Microbiological Associates, Inc., Bethesda, Md. 10^5 L1210 cells implanted ip, ip treatment.

A small number of naturally occurring or biosynthetic analogs of actinomycin D have been tested in in vivo assay systems for antitumor activity in our program. Table 14 shows comparative antitumor activity in four in vivo tumor systems for several analogs of actinomycin D which have modifications in the amino acid composition of the cyclic pentapeptide moieties. Actinomycin B and actinomycin C are mixtures of actinomycins. Actinomycin C had activity in L1210 leukemia and adenocarcinoma 755 similar to the activity of actinomycin D. This is not surprising since the components of actinomycin C, namely actinomycin D, actinomycin C_2, and actinomycin C_3, appear to have similar potency and efficacy in the L1210 and P388 leukemia systems. These results support findings in other systems which indicate that substitution of D-*allo*-isoleucine for D-valine in the 2-position of the cyclic pentapeptide chains does not markedly alter the biologic activity of the actinomycins (29). Actinomycin B had less activity in very limited testing than did actinomycin D.

TABLE 12.—Combination treatment of early ip L1210 leukemia with actinomycin D plus emetine*

Compound	Schedule	Optimal dose (mg/kg)	%ILS
Actinomycin D alone	qd, Days 1–9	0.064	58
Emetine alone	q4d; Days 1,5,9	15.0	54
Actinomycin D + Emetine	qd, Days 1–9 + q4d; Days 1,5,9	0.032 + 15.0	70
Actinomycin D alone	q4d; Days 1,5,9	0.30	26
Emetine alone	q4d; Days 1,5,9	16.0	37
Actinomycin D + Emetine	q4d; Days 1,5,9 (interval varied from 0 to 24 hrs) q4d; Days 1,5,9	0.30 + 16.0	47–79

*10^3 L1210 cells implanted ip, ip treatment.

TABLE 13.—Combination treatment of early ip L1210 leukemia with actinomycin D plus L-asparaginase*

Compound	Schedule	Optimal dose (mg/kg)	%ILS
Actinomycin D alone	qd, Days 1–9	0.032	32
Actinomycin D alone	q4d; Days 1,5,9	0.256	38
L-Asparaginase alone	qd, Days 1–6	7.5–30 IU	0
Actinomycin D + L-Asparaginase	qd, Days 1–9 + qd, Days 1–6	0.032 + 15 IU	29
Actinomycin D + L-Asparaginase	q4d; Days 1,5,9 + qd, Days 1–6	0.256 + 30 IU	34

*10^3 L1210 cells implanted ip, ip treatment.

Growth of *Streptomyces antibioticus* in medium supplemented with pipecolic acid (homoproline) results in biosynthesis of actinomycins with homoproline in place of proline in the 3-position of the cyclic pentapeptide chains of actinomycin D (30). Two of these derivatives, actinomycin $Pip_{1\beta}$, which has homoproline in place of proline in one of the peptide chains, and actinomycin Pip_2, which has homoproline in place of proline in both chains, have been tested in our program. Both compounds have activity in L1210 leukemia, though the optimal ILS is slightly less than that obtained with actinomycin D. Although these compounds have an efficacy (optimal ILS) similar to the parent compound, the doses required for antitumor activity are about six times the optimal dose of actinomycin D, indicating a considerable decrease in potency. It should be kept in mind that potency is of little importance in the evaluation of antitumor agents in in vivo tumor systems. It is the degree of antitumor activity that is the critical factor. However, actinomycin Pip_2 does appear to be inferior to actinomycin D in that it is less effective in the treatment of P388 leukemia or sarcoma 180.

TABLE 14.—Antitumor activity of naturally occurring actinomycin analogs

NSC No.	Actinomycin analog	L1210 leukemia		P388 leukemia		Adenocarcinoma 755‡		Sarcoma 180§	
		OD*	%ILS	OD†	%ILS	OD	%TWI	OD	%TWI
3053	Actinomycin D	0.05	45	0.05	>200	0.05	100	0.03	83
18268	Actinomycin C	0.02	42	—	—	0.05	88	—	—
25792	Actinomycin B	0.12	11	—	—	0.02	58	0.01	17
26742	Actinomycin E	0.50	17	—	—	0.25	6	0.25	7
48077	Actinomycin Pip_2	4.0	32	0.30	40	—	—	0.20	17
87221	Actinomycin C_2	0.05	47	0.05	>200	—	—	—	—
87222	Actinomycin C_3	0.05	46	0.10	>200	—	—	—	—
107660	Actinomycin $Pip_{1,2}$	0.31	36	—	—	—	—	—	—

*OD = optimal dose. All doses represent the OD on daily administration (Days 1–9) except for actinomycin Pip_2 which was tested as a single dose.

†OD on daily administration (Days 1–10).

‡OD on daily administration (Days 1–11); %TWI = percent tumor weight inhibition as measured on Day 12.

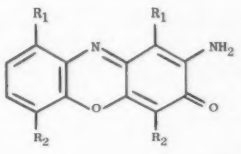
§OD on twice daily administration (Days 1–7); %TWI = percent tumor weight inhibition as measured on Day 8.

Actinomycin E, in which L-*allo*-*N*-methylisoleucine replaces L-*N*-methylvaline in the 5 position of the cyclic pentapeptide chain(s) and D-*allo*-isoleucine replaces D-valine in the 2-position (29), is inactive in L1210 leukemia, adenocarcinoma 755, and sarcoma 180.

The inactivity of several simplified synthetic actinomycin homologs (table 15) is evidence for the importance of the structure of the cyclic pentapeptide chains in the biologic activity of actinomycins. The experimental system used in this comparison is the in vitro cultured KB cell line of human epidermoid carcinoma. Actinomycin D is one of the most active agents in this in vitro growth inhibition assay, with a 50% inhibitory concentration in the picogram/ml range. None of these synthetic partial structures meets the criteria of activity in this system, which is an ED50 < 4 μg/ml.

In conclusion, the antitumor activity of actinomycin D has been studied extensively in our program. This agent has definite activity in a variety of experimental tumor systems although it appears to have a somewhat narrower spectrum of activity than several of the most useful clinical antitumor agents. The experimental results obtained with actinomycin D do not give any clear-cut indication of sched-

TABLE 15.—Activity of simplified actinomycin derivatives against cultured KB cells



NSC No.	R_1	R_2	Activity in KB cell culture (ED50, μg/ml)*
3053 (Actinomycin D)	L-NmeValine Sarcosine O L-Proline D-Valine L-Threonine C = O	$-CH_3$	1×10^{-5}
67108	O -C-glycine	$-H$	30
94945	$-H$	$-H$	16
96003	O -C-OnBu	$-CH_3$	10
407187	O -C-L-Threonine methyl ester	$-CH_3$	>100
407188	O -C-L-Threonine carboxamide	$-CH_3$	10

*ED50 = concentration of drug in the growth medium which inhibits growth by 50% by 3 days after drug addition.

ules of administration which could lead to improved therapeutic effectiveness through reduction of host toxicity or increased antitumor effect.

Although only a limited number of combinations have been studied, the data indicate that therapeutic synergism may be obtained with combinations of actinomycin D and other drugs. It will be important to carry out further studies on the therapeutic effectiveness in experimental systems of actinomycin D in combination with other clinically active antitumor agents.

Of the limited number of analogs which have been tested, none have shown therapeutic effectiveness superior to actinomycin D. However, actinomycins C_2 and C_3 appear to be as effective as the parent compound. A more extensive program of structure-activity analysis of actinomycins is warranted. Such studies may uncover analogs that could be considered for further development toward clinical use if they are clearly more effective than actinomycin D in such systems as L1210 leukemia and B16 melanoma or have a wider spectrum of activity in tumors which are not sensitive to the parent compound.

LIST OF COMPOUNDS

Actinomycin D: NSC-3053
Adriamycin: NSC-123127
L-Asparaginase: NSC-109229
Azaserine: NSC-742; CAS reg. No. 115–02–6; serine, diazoacetate (ester), L-
BCNU: NSC-409962; CAS reg. No. 154–93–8; urea, 1,3-bis(2-chloroethyl)-1-nitroso-
Bleomycin: NSC-125066
Chromomycin A_3: NSC-58514; CAS reg. No. 7059–24–7
Cyclophosphamide: NSC-26271; CAS reg. No. 6055–19–2; 2*H*-1,3,2-oxazaphosphorine, 2-[bis(2-chloroethyl)amino]tetrahydro-, 2-oxide, monohydrate; Cytoxan
Daunorubicin: NSC-83142; CAS reg. No. 23541–50–6; daunomycin (NSC-82151)
DON: NSC-7365; norleucine, 6-diazo-5-oxo-, L-
Emetine: NSC-33669; CAS reg. No. 316–42–7; emetine hydrochloride
5-Fluorouracil: NSC-19893; CAS reg. No. 51–21–8
Hydrocortisone: NSC-10483; CAS reg. No. 50–23–7
Mechlorethamine: NSC-762; CAS reg. No. 55–86–7; diethylamine, 2,2′-dichloro-*N*-methyl-, hydrochloride; nitrogen mustard
6-Mercaptopurine: NSC-755; CAS reg. No. 6112–76–1; purine-6-thiol, monohydrate; 6-MP
Methotrexate: NSC-740; CAS reg. No. 59–05–2; glutamic acid, *N*-[*p*-[[(2,4-diamino-6-pteridinyl)methyl]-methylamino]benzoyl]-
Mithramycin: NSC-24559; CAS reg. No. 18378–89–7
Mitomycin C: NSC-26980; CAS reg. No. 50–07–7
Neocarzinostatin: NSC-69856
Pactamycin: NSC-52947; CAS reg. No. 23668–11–3
Streptozotocin: NSC-85998
6-Thioguanine: NSC-752; CAS reg. No. 1832–72–0; purine-6-thiol, 2-amino-
Vinblastine: NSC-49842; CAS reg. No. 6449–03–2; vincaleukoblastine, sulfate (1:1), monohydrate
Vincristine: NSC-67574; CAS reg. No. 2068–78–2; leurocristine, sulfate (1:1)

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Possible Approaches to Further Actinomycin Studies

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In terms of its use as an antineoplastic agent, actinomycin presents a problem that is common to many drugs of its class: it is a very effective therapeutic agent against a very small number of malignant diseases, and it is almost totally useless against the vast majority of tumors. The biochemical basis of actinomycin action in eukaryotic cells seems to be well understood, and all of its short term effects are consistent with the accepted idea that, at dose levels such as those used in animals exposed to sublethal doses of antibiotic, actinomycin forms complexes with DNA and blocks the synthesis of RNA, particularly that of rRNA. Although this single mechanism appears to be uniformly true for mammalian cells, especially for cells in culture, there are wide variations in the ultimate consequences of actinomycin action in different cells. Thus, for equivalent degrees of inhibition of total RNA synthesis (eg, 99%), some cells (KB) will be lysed in 30–60 minutes and other cells (chick embryo fibroblasts) will lyse after 6–12 hours. In contrast, L-cells remain attached to the monolayer and retain metabolic activity and osmotic integrity for 2–3 days.

An extreme example of cell survival when RNA synthesis has been completely blocked by actinomycin is provided by the Vero monkey cell line, which quickly recovers from exposure to as much as 10 μg/ml for some hours and resumes both RNA synthesis and growth following a short lag period after the antibiotic is removed by washing. We do not know why some cells die after brief exposure to actinomycin and others survive, nor is there any evidence to suggest why the cytotoxic effects of the antibiotic are irreversible for some cells and reversible for others. Finally, there are indications that comparable variations both in cytotoxicity and in reversibility of actinomycin effects are characteristic of different cells in the body. A particularly striking example comes from the work of Schwartz et al (1) who reported that single intravenous injections of relatively high doses of actinomycin administered to the rat gave rise to a paralysis resulting from degeneration of the dorsal root ganglia and posterior columns. It is known that the levels of actinomycin used by Schwartz et al profoundly inhibit RNA synthesis in the liver for up to 24 hours, yet there is no liver degeneration and the hepatic cells survive and recover from the effects of the drug.

Another perplexing aspect of actinomycin pharmacology comes from a comparison of its properties with those of other antibiotics, such as daunorubicin and mithramycin,[2] whose biochemical mechanisms of action are identical to that of actinomycin; indeed, mithramycin even binds to the same base in DNA as actinomycin. Despite these biochemical similarities, the antitumor spectra of these drugs and their toxic effects in man are quite different.

While it is unlikely that drugs such as actinomycin can be converted into truly broad-spectrum antitumor agents, it seems reasonable to expect that they could be usefully applied to a larger number of malignancies than at present; moreover, their efficacy could be improved on the basis of additional information about their cellular effects. The kind of information required to broaden the scope and to optimize the application of actinomycin could be obtained from a careful definition of the pharmacokinetic properties of actinomycin at the cellular and tissue levels, and from a better appreciation of the factors that determine whether cell death occurs after actinomycin therapy. This type of knowledge can be expected to generate new treatment protocols

[1] Rockefeller University, New York, NY.

[2] Daunorubicin: NSC-83142; CAS reg. No. 23541-50-6.

Mithramycin: NSC-24559; CAS reg. No. 18378-89-7.

and to suggest new specific applications; these could also provide the improvements in therapeutic index required to achieve more successful use of actinomycin and related drugs. The chemistry, metabolism, and assay of actinomycin are very favorable for the performance of such studies.

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Potential of Actinomycin in Cancer Control

Irving H. Goldberg [1]

I should like to comment on four areas of actinomycin research that are in need of further exploration. In fact, I believe that these remarks have implications for chemotherapeutic research in general.

The question of what the cell does to actinomycin after the cell has taken up the drug and has allowed it to complex with nuclear DNA deserves special emphasis. We have heard a lot about the possible importance of the subsequent disposition of actinomycin once it is in some way released from the DNA and apparently extruded from the cell. I think this area has been understudied and deserves a great deal more attention. It already seems clear that this disposition process varies from cell type to cell type and that it is an important factor in determining how effective actinomycin is in a particular cell type. There may, in fact, be an avenue for exploration here in terms of greater effectiveness of therapy, especially as it may relate to some of the so-called inactive derivatives. If the process of binding of actinomycin to DNA depends on certain obvious parameters such as the precise nature of the chromophore and peptides, perhaps the system which is involved in the subsequent release from DNA and from the cell itself depends on some other characteristics of the molecule which can be mimicked in certain of the inactive derivatives, that is, molecules that are biologically inactive or that bind very poorly, if at all, to the DNA but yet may share some sort of a transport system with native actinomycin. If such a system in fact exists, administering a so-called inactive derivative might permit the cell to maintain the relatively high concentration of the active component for a much longer time. Whether there is any basis for such a possibility remains to be seen and will depend on a much more extensive study of the release phenomenon. The importance of the further study of this phenomenon cannot be overemphasized.

Some of Dr. Sidney Farber's original studies were on the increased radiosensitivity associated with the prior or subsequent administration of actinomycin. There is a great deal about this phenomenon that we don't yet understand and that we should study further. The phenomenon is obviously linked to the whole process of DNA repair, and I think that one can easily make a case for more extensive exploration of what seems to be an easily reproducible process about which we have relatively little information. From an additional study of this association, we can obtain much information on the precise mechanism of cell killing and repair of DNA which could be very useful from the therapeutic point of view.

Generally, in pharmacology we are often faced with situations where a particular drug seems to be effective in a relatively small percentage of instances, perhaps in only 5% or 10% of the cases. We usually focus on the instances where there is no effect and tend to forget the few instances when the effect is actually quite striking. Again, in the case of actinomycin in certain tumor types and in certain clinical situations, we should go into much greater depths of study of those particular instances of therapeutic effect even though they may be quite uncommon. These investigations will require more detailed analyses of pharmacokinetics and pharmacogenetics and should not in any way be limited just to animal studies. The time has come for an in-depth study of the clinical pharmacology of such agents in patients who have the disease that we are interested in and who provide the opportunity for studying a possible therapeutic effect.

The last comment I want to make relates to what we think we know about the mechanism of action of drugs like actinomycin, in the hope

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that we can perhaps design more effective agents. Actinomycin and most of the other drugs that have been alluded to so far at this meeting are agents which combine with DNA and exert their effects via this interaction. We also know about a group of compounds that affect DNA transcription by directly inactivating the RNA polymerase, such as α-amanitin and certain rifamycin derivatives. More recently, there has been an interest in drugs that appear to combine both kinds of action, that is, drugs that actually complex with the DNA but inactivate the RNA polymerase in this complex. Examples of this kind of agent are kanchanomycin (1–3), a very cytotoxic agent, and luteoskyrin (4), in which Fromageot et al have been interested.[2] Luteoskyrin is the principal element of yellowed rice which is thought to be important in producing tumors of the liver in the Far East. Once we have detailed information on the molecular mechanism of action of agents like kanchanomycin and luteoskyrin, we may have an opportunity to modify actinomycin or other intercalating agents which interact solely with the DNA so as to make them even more effective compounds.

[2] Kanchanomycin: NSC-62773.
Luteoskyrin: CAS reg. No. 21884-44-6.

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Controlled Biosynthesis of Actinomycins [1]

Edward Katz [2,3]

SUMMARY

Controlled or directed biosynthesis of the actinomycins has been achieved using amino acids or amino acid analogs that are chemically related to the amino acid constituents in actinomycin. As a consequence, there is enhanced formation of actinomycins normally produced in trace amounts or synthesis of new actinomycins. Exogenously provided compounds may be incorporated directly or they may be modified biochemically in a manner analogous to that for the natural amino acid components (eg, hydroxylation, oxidation, racemization, methylation). Thus far, the amino acids at positions 2, 3, and 5 of the actinomycin peptides have been replaced.

[Cancer Chemother Rep 58:83–91, 1974]

This paper represents a summation of results obtained by different laboratories as well as by our own group. The findings have already appeared in a number of publications elsewhere. I wish to describe some of the data concerned with the directed biosynthesis of these unusual peptide antibiotics.

Brockmann and Pfennig (1), Schmidt-Kastner (2–4), and our own laboratory (5,6) demonstrated that the composition of an actinomycin mixture synthesized by a given organism varies somewhat according to the conditions of cultivation. It became clear that the most important factor influencing antibiotic synthesis quantitatively and qualitatively was the nitrogen source (or sources) supplied to the organism.

Controlled or directed biosynthesis of the actinomycins has been achieved by using two nitrogen-containing compounds in the culture medium (2,4,7–9). One compound, which is present when the medium is inoculated with the organism, serves as a source of nitrogen for protein and nucleic acid synthesis and, hence, growth. The second nitrogen-containing compound, which is supplied when antibiotic synthesis begins, serves to direct or influence actinomycin formation. It has been found that the amino acids that function most effectively as "moderators" of antibiotic formation are (*a*) certain amino acids that are present in trace or minor actinomycin components and (*b*) those amino acids that are chemically similar to one of the amino acids in an actinomycin molecule. The exogenously supplied compound competes with the endogenously synthesized amino acid and is incorporated into the actinomycin peptides with either (*a*) enhanced formation of trace actinomycin components or (*b*) formation of new actinomycins. One striking feature of controlled biosynthesis is that the amino acid or amino acid analog provided

[1] Supported by Public Health Service grant CA-06926 from the National Cancer Institute, National Institutes of Health, Department of Health, Education, and Welfare.

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[3] The author wishes to acknowledge the contributions of the following investigators who have collaborated with him on various aspects of the studies concerned with the directed biosynthesis of the actinomycins: Drs. William Goss, Joseph V. Formica, Tadashi Yoshida, Yasuo Kawai, and Anthony Mauger, Mr. Takehiko Yajima, Mrs. Mary Ann Grigg, and Miss Nora Krapohl.

is frequently modified biochemically (eg, via hydroxylation, oxidation, or methylation reactions) in the same manner as the natural amino acid.

VALINE AND ISOLEUCINE STUDIES

Amino acid substitutions have been observed at several sites in the actinomycin molecule (*N*-methylvaline, D-valine–D-*allo*-isoleucine, or proline sites). For example, Schmidt-Kastner (2) demonstrated that the amount of actinomycin IV in the mixture synthesized by *Streptomyces chrysomallus* increased from 10% to 83% when DL-valine was added to a chemically defined medium.[4] Similar results (10) were obtained in our laboratory (table 1).

Schmidt-Kastner (2,3) also noted that new actinomycins were synthesized when DL-isoleucine was supplied to *S. chrysomallus* or certain other actinomycin-producing organisms. Two new actinomycins, designated E_1 and E_2, were synthesized by *S. chrysomallus* in the presence of DL-isoleucine. It was reported (2,3) that actinomycin E_1 contained one residue of *N*-methylisoleucine and that actinomycin E_2 contained two residues of *N*-methylisoleucine, replacing one and two molecules of *N*-methylvaline, respectively. D-Isoleucine proved to be more effective than L-isoleucine for the synthesis of actinomycins E_1 and E_2 (10). Recent studies have been carried out with the four stereoisomers of isoleucine (fig 1) (11,12). In experiments with *Streptomyces antibioticus*, it was observed that D-*allo*-isoleucine and D-isoleucine were quite inhibitory to antibiotic formation, whereas L-isoleucine and L-*allo*-isoleucine were only slightly inhibitory. Amino acid hydrolysates of actinomycin mixtures synthesized by *S. chrysomallus* and *S. antibioticus* in the presence of L- or D-isoleucine or L-*allo*-isoleucine contained isoleucine and *N*-methyl-*allo*-isoleucine but not *N*-methylisoleucine.

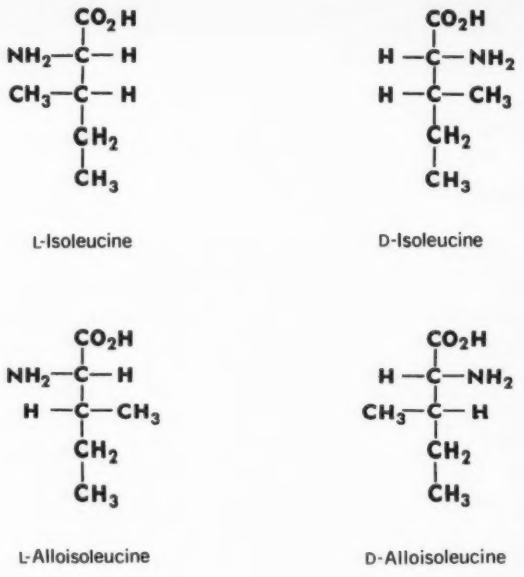

FIGURE 1.—Structure of isoleucine stereoisomers.

Figure 2 shows the separation of amino acids that can be achieved using a two-dimensional paper electrophoretic-chromatographic technique. Quantitative analyses performed with the amino acid analyzer revealed that there were significant amounts of *N*-methyl-*allo*-isoleucine, isoleucine, and even *allo*-isoleucine in the hydrolysates (table 2). Isoleucine and *N*-methyl-*allo*-isoleucine were isolated and purified from the actinomycin mixtures formed in the presence of D-isoleucine by *S. antibioticus* (12). The homogeneity and identity of the amino acids were established with standard amino acids by high-voltage electrophoresis as well as by paper and column chromatographic procedures. The optical configuration of the amino acids was determined by optical rotatory dispersion. It was established that *N*-methyl-*allo*-isoleucine exhibited a positive Cotton effect; in contrast, isoleucine exhibited a negative Cotton effect. These results indicate that the *N*-methylamino acid has the L- configuration and that isoleucine has the D- configuration. The D- configuration of isoleucine was also confirmed by means of D- and L-amino acid oxidase studies

[4] Actinomycin designations by Roman numerals are those proposed by Waksman et al in: Waksman SA, Katz E, and Vining LC. Nomenclature of the actinomycins. Proc Natl Acad Sci USA 44:602–612, 1958. They correspond as follows to the letter designations proposed by Brockmann (*see* paper by Hans Brockmann in this issue, pp 9–20): I = $X_{0\beta}$; II = A_{II}; III = $X_{0\gamma}$, F_0; IV = C_1, D; V = X_2; VI = C_2; VII = C_3.

TABLE 1.—Influence of D-, L-, and DL-valine on actinomycin synthesis by *Streptomyces chrysomallus**

Valine isomer added (optical configuration)	Amount added (μg/ml)	Actinomycin component in mixture (%)†		
		IV	VI	VII
None	0	19.0	41.8	39.2
L-	10	14.4	42.4	43.2
D-	10	19.6	41.3	39.1
DL-	20	13.6	44.2	42.2
L-	250	24.2	36.7	39.1
D-	250	15.6	43.3	41.2
DL-	500	42.3	31.7	26.0
L-	500	33.9	30.2	35.9
D-	500	18.3	33.9	47.8
DL-	1000	53.2	20.9	25.9
L-	1000	58.8	28.1	13.1
D-	1000	16.0	37.8	42.2
DL-	2000	71.1	18.3	10.7

*The organism was grown in glutamic acid-galactose medium for 24 hours at which time D-, L-, or DL-valine was added. After an additional incubation period of 48 hours, the actinomycin mixture formed was isolated from culture filtrates and separated by circular paper chromatography. The relative percentage of a component in a given mixture was determined as described previously (10). [From Katz et al (10). Reprinted with permission from the American Society of Microbiology.]

†Actinomycin IV = C_1, D; VI = C_2; VII = C_3. (*See* footnote 4 in text.)

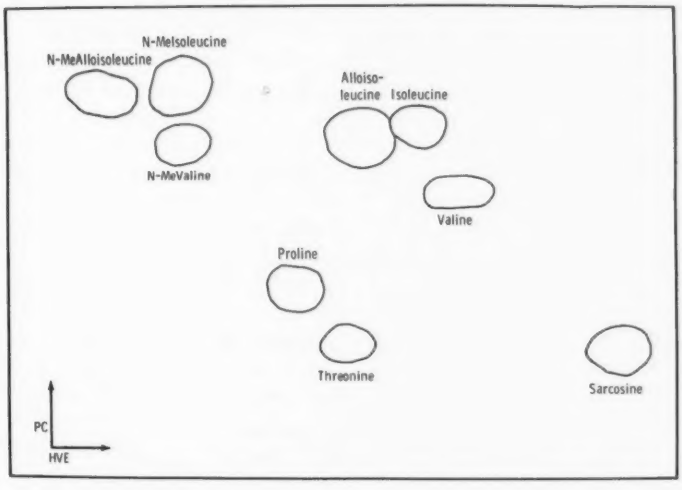

FIGURE 2.—Separation of amino acids by two-dimensional paper electrophoresis and chromatography. [From Yajima et al (12). Reprinted with permission from Academic Press, Inc.]

and by the preparation of diastereoisomeric L-leucyl dipeptides.

It was concluded (12) that D-isoleucine and *N*-methyl-L-*allo*-isoleucine replace D-valine and *N*-methyl-L-valine, respectively, in the actinomycin peptides that are synthesized by *S. antibioticus* and *S. chrysomallus* in the presence of one of the isoleucine stereoisomers. The possible interrelationships of these amino acids in the biosynthesis of the actinomycins are shown in figure 3.

SARCOSINE STUDIES

It has also been reported (2–4) that, in addition to actinomycins C_1, C_2, and C_3, several new sarcosine-rich actinomycins (designated F) are formed when *S. chrysomallus* is grown in a medium containing sarcosine. Analyses of pure actinomycin components suggested that exogenously provided sarcosine substituted for endogenously formed proline in the antibiotic peptides. Actinomycins F_8 and F_9 are derivatives of C_1, actinomycins F_1 and F_2 are considered to be derivatives of C_2, and actinomycins F_3 and F_4 are considered to be derivatives of C_3 (4,13).

Katz and Goss (5,9,14,15) studied the influence of sarcosine on actinomycin formation by *S. antibioticus*. Actinomycins II and III, which are normally produced in trace amounts (2%–4%), increased to approximately 60%–65% of the mixture synthesized in the presence of sarcosine (table 3). In addition, one new component (which has not been characterized) was synthesized. As the production of actinomycins II and III increased, the synthesis of actinomycin IV was greatly decreased. It was postulated that exogenously supplied sarcosine competed with endogenously formed proline and that it was incorporated in lieu of proline into certain actinomycin peptides. As a consequence, actinomycins II and III were formed instead of actinomycin IV (14). Johnson and Mauger (16), as well as our own group (6,15), demonstrated that actinomycins II and III both contain the amino acids valine, threonine, sarcosine, and *N*-methylvaline. They differ qualitatively and quantitatively in that actinomycin II possesses four residues of sarcosine but no proline whereas actinomycin III possesses three residues of sarcosine and one residue of proline per molecule of actinomycin.

PROLINE ANALOGS

Because competition between sarcosine and proline appears to be responsible for the observed shift in antibiotic synthesis, numerous analogs of proline have also been examined as potential replacements for proline in the actinomycin peptide. With pipecolic acid, the higher analog of proline, several new actinomycins representing at least 50% of the total actinomycin mixture were synthesized by *S. antibioticus*

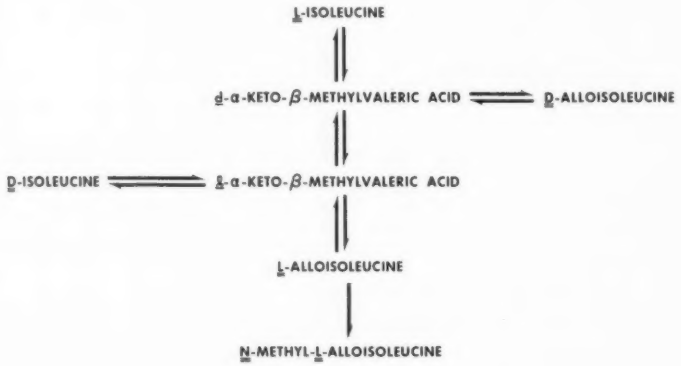

FIGURE 3.—Possible mechanism for the synthesis of *N*-methyl-L-*allo*-isoleucine and D-isoleucine from the various isomers of isoleucine. [From Yajima et al (12). Reprinted with permission from Academic Press, Inc.]

TABLE 2.—Analysis of *N*-methyl and branched-chain amino acids in hydrolysates of actinomycin preparations*

Stereoisomer added to medium	Amount added (μg/ml)	Concentration of amino acid (moles/mole of actinomycin)				
		N-Methyl-L-valine	*N*-Methyl-L-*allo*-isoleucine	D-Valine	D-*allo*-Isoleucine	D-Isoleucine
S. chrysomallus						
None	0	2.00	0.00	0.87	1.13	0.00
L-*allo*-Isoleucine	100	0.70	1.30	0.29	0.97	0.73
D-Isoleucine	100	1.08	0.92	0.70	0.69	0.61
L-Isoleucine	500	1.25	0.75	0.14	1.68	0.17
D-*allo*-Isoleucine	100	1.98	0.02	0.94	1.01	0.07
S. antibioticus						
None	0	1.77	0.23	2.00	trace	trace
L-*allo*-Isoleucine	100	0.84	1.16	0.55	0.12	1.33
D-Isoleucine	100	1.03	0.97	0.83	0.10	1.07
L-Isoleucine	500	0.59	1.41	0.70	0.19	1.11
D-*allo*-Isoleucine	100	1.23	0.77	1.92	0.06	0.02

**Streptomyces chrysomallus* and *Streptomyces antibioticus* were cultivated in glutamic acid-galactose medium. After a 24-hour incubation period, one of the four stereoisomers of isoleucine was added and the incubation was resumed for a 48–96-hour period. Hydrolysates of actinomycin mixtures were examined on the Beckman Spinco amino acid analyzer (Model 120C) using 0.2 M sodium citrate buffer (pH 3.05 and 4.25). [From Yajima et al (12). Reprinted with permission from Academic Press, Inc.]

(14,17,18). When azetidine-2-carboxylic acid, the lower analog of proline, was used, at least two, and possibly three, new actinomycins were formed (5). Synthesis of new actinomycins has also been observed when either of these analogs is supplied to *S. chrysomallus* or other actinomycin-forming organisms. Several other proline analogs also appear to direct the synthesis of new actinomycins. Evidence has been obtained that 4-methylproline and, to a limited extent, 5-methylproline are incorporated into the actinomycin molecule in lieu of proline (19, 20). Haloprolines (6) such as 4-fluoro-, 4-bromo-, and 4-chloroproline, and even thiazolidine-2-carboxylic acid (thioproline) (21), can also substitute for proline. A summary of the effects of various amino acids and amino acid analogs on actinomycin synthesis is shown in table 4.

PIPECOLIC ACID-CONTAINING ACTINOMYCINS

We have recently completed studies on the pipecolic acid-containing actinomycins synthesized by *S. antibioticus* (17,18). Six compounds were separated and purified from antibiotic mixtures formed in the presence of this imino acid analog. The compounds have been designated actinomycins Pip 2, Pip 1α, Pip 1β, Pip 1γ, Pip 1δ, and Pip 1ε. The amino acid composition of these antibiotics was established by a combination of high-voltage electrophoresis and various chromatographic procedures. Each of the new actinomycins contains two residues of threonine, valine, *N*-methylvaline, and sarcosine. They differ in imino acid content as shown in table 4. The presence of pipecolic acid, 4-oxopipecolic acid, and *trans*-4-hydroxypipecolic acid (fig 4) was confirmed by cochromatography and coelectrophoresis with authentic standards of these imino acids. Also, a comparison of hydrolysates of vernamycin Bα (22) and ostreogrycin (23) (peptide antibiotics known to contain 4-oxopipecolic acid) revealed that the oxoimino acid was present in the Pip 1α and Pip 1δ actinomycins. 4-Oxopipecolic acid was reduced with sodium borohydride to a

TABLE 3.—Influence of sarcosine concentration on actinomycin biosynthesis by *Streptomyces antibioticus**

Sarcosine concentration (μg/ml)	Actinomycin component in mixture (%)†				
	I	II	III	IV	V
None	6.2	2.1	2.8	80.4	8.4
10.0	5.3	9.2	11.9	63.5	10.1
100.0	8.1	22.5	33.1	30.8	5.5
250.0	8.9	25.4	35.3	24.6	5.7

*Sarcosine was added to the medium after *S. antibioticus* had been incubated for 30 hours. After an additional 6 days of incubation, the actinomycin mixture was recovered and separated by circular paper chromatography, and the percentage of the actinomycin components in a mixture was determined. [From Katz (5). Reprinted with permission from the New York Academy of Sciences.]

†Actinomycin I = $X_{0\beta}$; II = A_{II}; III = $X_{0\gamma}$, F_0; IV = C_1, D; V = X_2. (*See* footnote 4 in text.)

mixture of *cis*-4-hydroxypipecolic acid (93%) and *trans*-4-hydroxypipecolic acid (7%); this agrees with the findings of Clark-Lewis and Mortimer (24). Finally, optical rotatory dispersion studies showed that the imino acids possess the L- configuration.

The effect of the six pipecolic acid-containing actinomycins on the *Escherichia coli* DNA-dependent RNA polymerase reaction was examined also (fig 5). The data correlate well in that the actinomycins containing one proline residue were found to be more inhibitory than those containing a pipecolic acid residue instead of proline. Moreover, the actinomycins that contain hydroxypipecolic acid (Pip 1ϵ, Pip 1γ) exhibited the lowest activity for each series of compounds. The order of activity observed was as follows: actinomycin IV = Pip 1β > Pip 1δ > Pip 1ϵ > Pip 2 > Pip 1α > Pip 1γ.

Normally, *S. antibioticus* synthesizes proline from glutamic acid as demonstrated in a large number of micro-organisms. Proline is incorporated directly into actinomycin peptides or it is modified via hydroxylation or oxidation

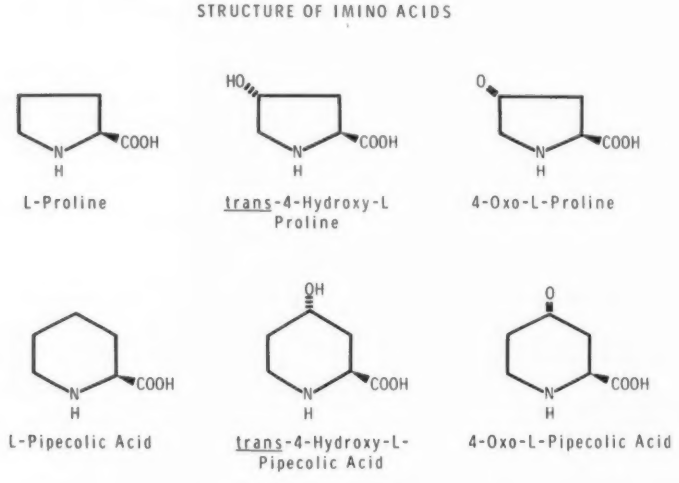

FIGURE 4.—Structure of imino acids.

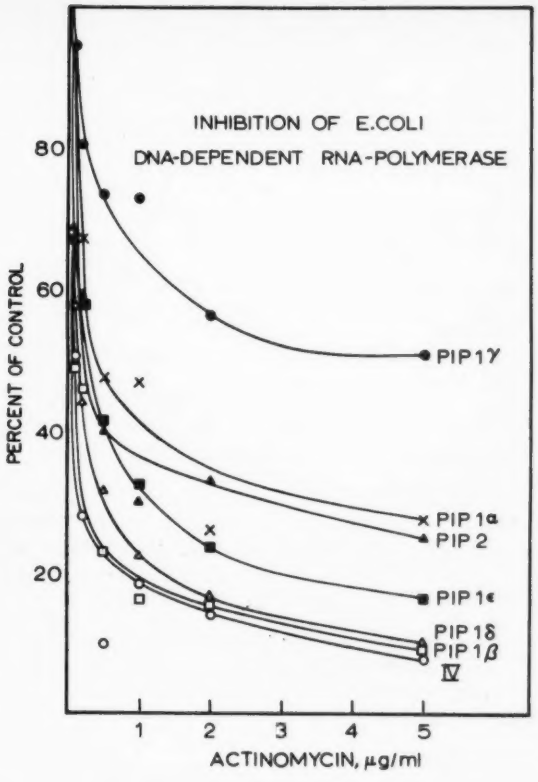

FIGURE 5.—Inhibition of the *Escherichia coli* DNA-dependent RNA polymerase reaction by various actinomycin preparations.

reactions to yield *trans*-4-hydroxy-L-proline (actinomycin I) or 4-oxo-L-proline (actinomycin V), respectively (25–27). When supplied with the proline analog pipecolic acid, the organism catalyzes similar biochemical reactions (fig 6). The utilization of these compounds for antibiotic biosynthesis results in the formation of novel actinomycins with quantitatively differing biological properties.

DISCUSSION

From the previous discussion, it is evident that exogenous or "foreign" amino acids can be incorporated directly into certain actinomycin peptides or they can be incorporated after modification by the antibiotic-producing microorganism.

To what extent can an actinomycin molecule be modified or altered? For example, can any one of the amino acids in the antibiotic peptide be replaced? Can two or more amino acids be replaced simultaneously? How specific are the enzymes involved in antibiotic peptide synthesis? The information presently available from the studies described here as well as those from other laboratories (28–32) suggests that the specificity requirements of the incorporating enzymes may not be of a particularly high order. In some cases, the exogenous compound bears a close structural relationship to the endogenously

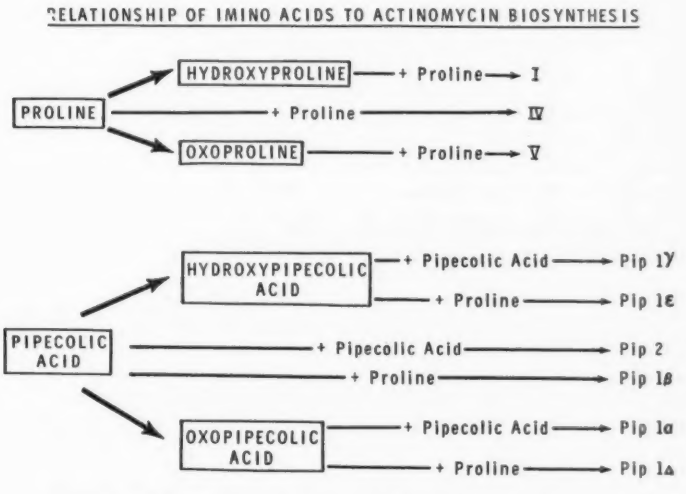

FIGURE 6.—Relationship of imino acids to actinomycin biosynthesis.

TABLE 4.—Influence of exogenous compounds on actinomycin synthesis

Site in peptide affected	Exogenous compound	Actinomycin component*	Percent of total actinomycin mixture	
			Before addition of exogenous compound	After addition of exogenous compound
Proline	Hydroxyproline	Actinomycin I	7	35
	Sarcosine	Actinomycins II + III	5	65
	Pipecolic acid	Six new components†: Pip 2, Pip 1β, Pip 1α, Pip 1γ, Pip 1δ, Pip 1ϵ	—	50
	Azetidine-2-carboxylic acid; 4- or 5-methylproline; 4-fluoro-, 4-bromo-, 4-chloro-, or 4-thioproline	New components		
D-Valine–D-*allo*-isoleucine, *N*-methyl-L-valine	L-Valine	Actinomycin IV	10	85
	L-Isoleucine, D-Isoleucine, L-*allo*-Isoleucine	New components containing *N*-methyl-L-*allo*-isoleucine and D-isoleucine		

*Actinomycin I = $X_{o\beta}$; II = A_{II}; III = $X_{o\gamma}$, F_o; IV = C_1, D. (*See* footnote 4 in text.)

†Imino acid content was as follows:

Actinomycin component	Imino acid content (No. of residues)			
	L-Proline	Pipecolic acid	4-Oxo-L-pipecolic acid	*trans*-4-Hydroxy-L-pipecolic acid
Pip 2	0	2	0	0
Pip 1β	1	1	0	0
Pip 1α	0	1	1	0
Pip 1γ	0	1	0	1
Pip 1δ	1	0	1	0
Pip 1ϵ	1	0	0	1

formed amino acid that it competes with (for example, valine with *allo*-isoleucine and azetidine-2-carboxylic acid or 4-methylproline or pipecolic acid with proline). In other instances, only a portion of the exogenous amino acid is similar (sarcosine versus proline).

The number of new compounds synthesized in any given situation will vary with (*a*) the antibiotic-producing organism, (*b*) the amino acid, (*c*) the concentration used, (*d*) the extent to which the exogenously provided compound is modified or metabolized, etc. A priori, one might expect several new actinomycins to be synthesized whenever an amino acid is incorporated. Controlled biosynthesis has been defined as the technique of predetermining the structure of a new antibiotic by furnishing specific chemical precursors to the antibiotic-producing micro-organism (33). This technique has been successfully applied to the formation of modified congeners of actinomycin.

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Actinomycin Monolactones: Origin and Importance [1]

D. Perlman [2]

SUMMARY

Actinomycin monolactone(s) isolated from actinomycin-producing streptomycete fermentations, from enzymic degradation of actinomycin by *Actinoplanes missouriensis* enzymes, and from mammalian cell cultures inhibited by actinomycin appear to have a metabolic role. Although the actinomycin monolactone is of very low cytotoxicity, combinations of actinomycin with monolactone resulted in potentiation of cytotoxicity.

[Cancer Chemother Rep 58:93–95, 1974]

Our recent studies (1–3) have shown that actinomycin monolactone (fig 1) can occur as a biosynthetic product of actinomycin-producing streptomycetes and as a degradation product formed from actinomycin by induced enzymes isolated from *Actinoplanes missouriensis*. The role of the actinomycin monolactone in the biosynthesis of actinomycin (2) is still unclear. Although it may be an intermediate under certain conditions, it can also be formed independently of actinomycin. In addition, it can occur as an inactivation/degradation product in microbial cultures and in tissue cultures which have been exposed to actinomycin.

Microbial Biosynthesis of Actinomycin Monolactones

In the course of our studies of actinomycin biosynthesis by *Streptomyces antibioticus* 3720, we noted that significant quantities of actinomycin monolactone and actinomycinic diacid could be recovered from the fermented media (1). Further investigations designed to show whether actinomycin monolactone(s) may be intermediate(s) in actinomycin biosynthesis, as previously concluded (2), are summarized in figure 2. It is obvious that if the streptomycete is grown under the conditions described in our previous study (1), actinomycin monolactone biosynthesis can proceed independently of actinomycin biosynthesis. Moreover, if the temperature of the incubator is raised (fig 2) or if iron salts are added to the medium, the amount of actinomycin produced is markedly reduced while the amount of actinomycin monolactone(s) formed is not affected. A semiquantitative chemical analysis was used for the measurement of the monolactone(s) based on separation by ionophoresis (4) followed by spectrophotometric measurement. In all of these studies we could not decide which of the monolac-

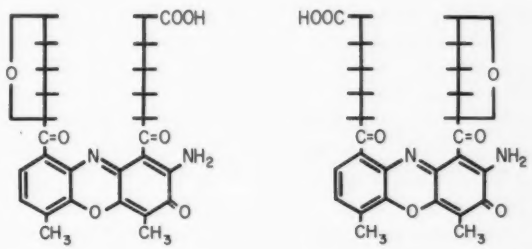

FIGURE 1.—Structures of actinomycin monolactones.

[1] Supported in part by the Graduate School Research Fund, University of Wisconsin; a grant from Eli Lilly and Company; and Public Health Service research grant AI-09320 from the National Institutes of Allergy and Infectious Diseases, National Institutes of Health, Department of Health, Education, and Welfare.

[2] School of Pharmacy, University of Wisconsin, Madison.

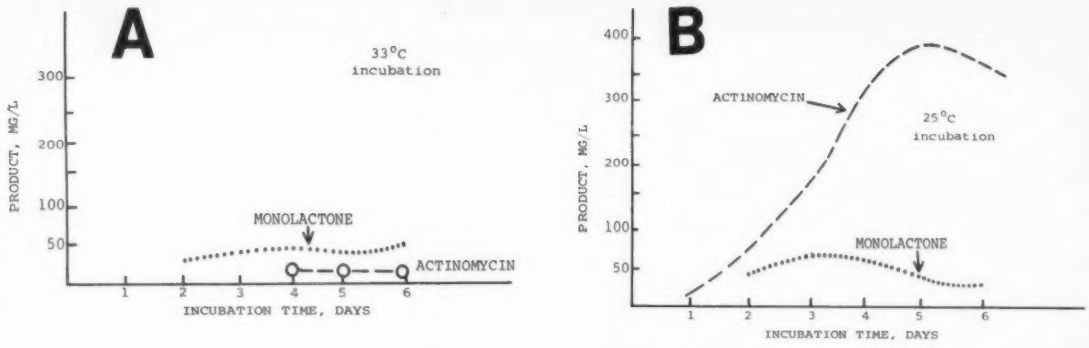

FIGURE 2.—Effect of incubation temperature on actinomycin and actinomycin monolactone formation by *S. antibioticus* 3720. A: 33 C; B: 25 C.

tones predominated, and the modified chemical method developed for this analysis (5) was not sensitive enough to be applied to the milligram amounts of material available from the fermentations.

In a series of related experiments, we added actinomycin monolactone prepared by enzyme hydrolysis of actinomycin (3,4) to an actinomycin-producing fermentation and did not observe evidence that the added material was converted to actinomycin. In fact, when actinomycin monolactone-^{3}H (prepared by enzyme hydrolysis of actinomycin D-^{3}H) was added to the growing culture, most of the radioactivity was not absorbed by the *S. antibioticus* 3720 cells. That which was absorbed (about 10% of the added material) was converted to actinomycin-^{3}H (identified by paper ionophoresis and column chromatography on LH-20 Sephadex), but this occurred only after extended incubation periods. Repetition of this experiment, using an actinomycin monolactone-^{3}H mixture prepared by chemical hydrolysis of actinomycin D (NSC-3053) (4), was equally disappointing, though it did indicate that neither of the two monolactones served as intermediates. (We assumed that the chemical hydrolysis of actinomycin D [4] resulted in an equimolar mixture of the monolactones.)

Bioactivity of Actinomycin Monolactone

Our previous report (4) mentioned that actinomycin monolactone(s) were essentially noncytotoxic to growing Earle's L_{929} cells in suspension culture. Since actinomycins were among the most cytotoxic compounds noted in this test system (6), we were interested in determining whether mixtures of monolactone and actinomycin might have increased toxicity over the actinomycin component alone. This interest was sparked by our finding that aqueous solutions of actinomycin D (50 μg/ml) underwent hydrolysis to a significant extent when stored in the dark at room temperature for 3 days. Some of the results of studies on the possible enhancement of actinomycin D cytotoxicity by the addition of monolactone are summarized in table 1. It seems that when the amount of actinomycin monolactone reached 25% of the total actinomycin-related material, enhancement of toxicity was noted. This may have some bearing on the dose regimens used in animal experimentation with actinomycin D,

TABLE 1.—Effect of actinomycin monolactone(s) on actinomycin D cytotoxicity*

Amount of actinomycin monolactone(s) added to basal medium (ng/ml)	ID50 for actinomycin D (ng/ml)
None	3
100	0.05
10	0.1
1	1
0.1	3

*Test system: Earle's L_{929} cells in suspension culture (6).

and also suggests the need for pharmacologic studies in which some differentiation of actinomycin and actinomycin monolactone is made in the various tissues and target organs.

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Origins of Base Specificity in Actinomycin and Other DNA Ligands

Donald M. Crothers [1] and Werner Müller [2]

SUMMARY

We review some important properties of the actinomycin-DNA complex and then focus on the origin of specificity for hydrogen-bonded guanine-cytosine base pairs. We suggest that electronic interactions between the intercalated chromophore and the G·C base pair provide an important contribution to this specificity. This view is supported by correlation of specificity with electronic factors in a series of simple intercalating agents. Finally, we suggest some important future applications for base-specific and sequence-specific DNA ligands.

[Cancer Chemother Rep 58:97–100, 1974]

The object of this presentation will be to show how study of the properties of actinomycin can be used as a basis for the design of new compounds potentially useful in cancer chemotherapy. We begin with a review of our findings from an earlier study (1) on the interaction of actinomycin with DNA. These fall under three general headings: structural aspects, kinetic properties, and base-specificity effects.

STRUCTURAL ASPECTS

The main structural feature established by our earlier experiments was intercalation of the actinomycin chromophore. Several experiments led to this conclusion; one of the simplest was the variation of the sedimentation coefficient of rod-like pieces of DNA when actinomycin was added. Since the sedimentation coefficient of a rod depends on the mass per unit length, it is simple to distinguish ligands that lengthen DNA from those that do not. Actinomycin was found to belong to the former class. This discovery led directly to an intercalation model in which the chromophore, inserted between adjacent base pairs, lengthens the double helix.

We proposed (1) a model in which the actinomycin peptide rings interacted with the small groove of the double helix. This model has since been displaced as the most reasonable by the model proposed by Sobell and coworkers (2), which is based on the crystal structure of the actinomycin-deoxyguanosine complex. Their model is similar to ours in general outline, but it is quite different in the detailed structure of the peptide rings. The final word on the structure of the actinomycin-DNA complex must await resolution of the crystal structure of the complex of the drug with a double-helical section of DNA.

KINETIC PROPERTIES

We found that the kinetic properties of the actinomycin-DNA complex are of particular importance for the biologic activity. For example, the compound actinomine (structure I, fig 1) binds to DNA as tightly as actinomycin does, but it is orders of magnitude less effective in blocking the action of RNA polymerase in vitro. In addition, a definite difference between the kinetic properties of actinomycin and the kinetic properties of actinomine was found. Whereas the actinomycin-DNA com-

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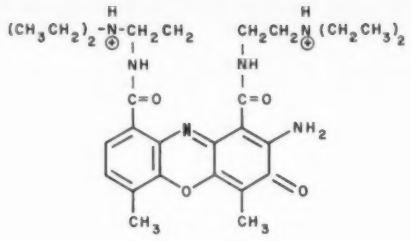


I

FIGURE 1.—Structure I (actinomine).

plex requires hundreds of seconds to dissociate, the actinomine-DNA complex dissociates in a fraction of a second. We rationalized this behavior with a model in which the RNA polymerase is delayed in its progress along the DNA template by the time it must wait for the drug to dissociate. Since actinomine dissociates much more rapidly, it is much less effective than actinomycin at an equal degree of binding. According to this model, any drug that is highly efficient in blocking the growth of mRNA chains by binding to DNA must have a long-lived DNA complex.

BASE-SPECIFICITY EFFECTS

Our equilibrium experiments also dealt with the origin of specificity for hydrogen-bonded guanine-cytosine base pairs. Consider a simple model for DNA-ligand interactions in which there are B_0 potential binding sites per base pair, each with an intrinsic binding constant K_0. When binding equilibrium data are treated according to the Scatchard method (3), r/m is plotted versus r, where r is the number of drug molecules bound per base pair and m is the concentration of free drug in solution. The intercept of the isotherm on the r/m axis is $B_0 K_0$ (4). We found (1) that for DNA of differing G + C content, $B_0 K_0$ varies approximately linearly with f_{GC}, the fraction of of G · C base pairs. Thus, if it is assumed that K_0 is a constant, then B_0 is proportional to f_{GC}. This leads to a simple model in which one G · C base pair is required for a binding site. (If binding to a particular sequence [such as two successive G · C base pairs] is preferred, then B_0 should vary with f_{GC}^2.)

The central question here is the origin of the preference for a single G · C base pair. Sobell and his colleagues (2) have emphasized the role of hydrogen bonding by the peptide side chains in establishing specificity. They consider that the principal binding site contains a GpC sequence in each strand, or two G · C base pairs. Given the twofold symmetry of their model, one would expect that the second G · C base pair would contribute as much to the specificity as does the first. However, the data indicate that although the first G · C base pair is crucial for binding, the binding constant is relatively indifferent to the presence of the second G · C base pair.

In our earlier paper (1), we suggested that specificity arises from electronic interactions between a polarized form of the chromophore and the relatively polar G · C base pair. In our view, it remains plausible that the major contribution to G · C base pair specificity comes from this source.

Supporting evidence for this view comes from our more recent studies on base specificity exhibited by intercalating ligands. We have prepared a series of dyes of the general form II shown in figure 2. The base specificity of these materials can be investigated by differential dialysis in which two DNAs of different G + C content are dialyzed against the same solution of free dye. The dye partitions itself according to its base preference so that r differs for the two DNAs. We let

$$\alpha = \lim_{m \to 0} \frac{r\ (Micrococcus\ luteus)}{r\ (Bacillus\ subtilis)}.$$

Furthermore, it is clear from the definition of $K_0 B_0$ given earlier that

$$\alpha = \frac{\Sigma\, K_0 B_0\ (M.\ luteus)}{\Sigma\, K_0 B_0\ (B.\ subtilis)},$$

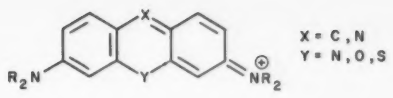
where the summation is included to take account of different kinds of binding sites.

We have found that specificity depends on the electronic properties of the chromophore.[3]

[3] Müller W, and Crothers DM. The effect of heteroatoms and polarizability on the base specificity of intercalating ligands. Paper in preparation.

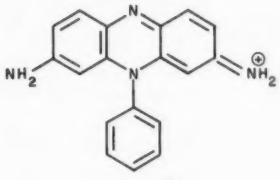
For example, in the series of compounds II (fig 2) with R = H, α increases as the absorbance maximum of the chromophore moves to a longer wavelength.

Specificity also depends on steric factors. This may be seen by comparing α values for compounds III–VI (fig 3),[4] which differ only in the placement of methyl groups.



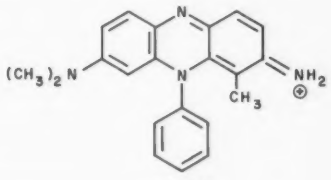
II

FIGURE 2.—Structure II (general form for a series of dyes).



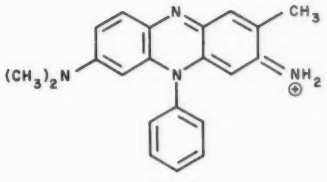
α = 1.45

III



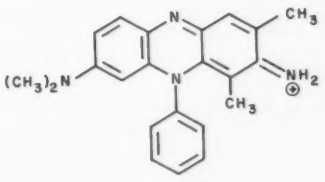
α = 1.70

IV



α = 1.71

V



α = 2.5

VI

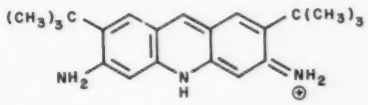
FIGURE 3.—Compounds III–VI.

For the two compounds with α = 1.7, $\Sigma K_0 B_0$ is proportional to f_{GC}, so that they are as specific for G • C base pairs as actinomycin is. Hence, base specificity can arise without special hydrogen bonding functions. The detailed nature of the steric factors that influence specificity is still unknown, however.

Most intercalating agents that show base specificity prefer G • C base pairs. Pronounced specificity for hydrogen-bonded adenine-thymine base pairs can be found for molecules that bind to the outside of the double helix. One example is the di-*tert*-butyl derivative of proflavine (structure VII, fig 4), which we have studied in detail (5). In this case, a binding site contains two A • T base pairs. The origin of this specific requirement is unknown.

COMMENT

What is the prospect for useful application of knowledge about base-specific interactions?



VII

FIGURE 4.—Structure VII (di-*tert*-butyl derivative of proflavine).

[4] Müller W, Bunemann H, and Datagupta N. Steric and electronic effects on the base specificity of intercalating ligands. Paper in preparation.

Some laboratory applications have already been realized. For example, compound V (phenyl neutral red) (fig 3) has been used in conjunction with hydroxyapatite chromatography to fractionate DNA from eukaryotic cells on the basis of G + C content (6). Our long-range interest is to combine G · C and A · T base-pair-specific residues on a polymer lattice so that specificity for base sequence can be achieved. We hope, for example, to be able to produce substances with a strong preference for binding to viral DNA over host DNA so that growth of the virus can be specifically repressed. Such materials could ultimately have important clinical applications.

In summary, our study of the properties of actinomycin launched us on a search for the principles of base specificity, which we propose to use to achieve base-sequence specificity. We are convinced that molecules that are much more selective in their action than actinomycin can be produced by vigorous pursuit of these principles.

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The Stereochemistry of Actinomycin Binding to DNA [1,2]

Henry M. Sobell [3]

SUMMARY

We have successfully cocrystallized actinomycin with its DNA substrate deoxyguanosine, and we have solved the three-dimensional structure of the complex by X-ray crystallography. The configuration observed in the crystalline complex explains in a natural way the stereochemistry of actinomycin binding to double-helical DNA. The phenoxazone ring system of actinomycin intercalates between the base-paired dinucleotide sequence dG-dC, while the peptide subunits lie in the narrow groove of the DNA helix and interact with deoxyguanosine residues on opposite chains through specific hydrogen bonds. The binding of actinomycin to DNA demonstrates a general principle that several classes of proteins utilize in recognizing symmetrically arranged nucleotide sequences on the DNA helix.

[Cancer Chemother Rep 58:101–116, 1974]

Actinomycin C_1 (D) (fig 1) is a cyclic polypeptide-containing antibiotic that is widely used in the study of molecular and cell biology. It is one of the most potent antitumor agents known; however, because of its extreme toxicity, it has had only limited clinical use. The activity of the antibiotic stems from its ability to bind double-helical DNA and inhibit RNA synthesis specifically (1–6). Studies with naturally occurring and synthetic DNA duplexes (4,6,7) have shown that deoxyguanosine residues are essential for the binding reaction. The spectral perturbations that accompany actinomycin binding to DNA can be mimicked by the addition of deoxyguanosine to solutions of actinomycin (8) although similar spectral changes can be induced with a variety of other purines and organic aryl sulfonates (6,9).

Two models have been proposed to explain the binding of actinomycin to DNA. The first model involves direct hydrogen-bonding recognition between the guanine ring and the chromophore residue. It is based on studies with a series of synthetic polymers possessing or lacking 2-amino functional groups on purine residues as well as on careful characterization of the chemical groups on actinomycin that are essential for binding (10–12). The second model, which is based on detailed spectroscopic, hydrodynamic, and kinetic measurements and supported by observations that actinomycin promotes the unwinding of supercoiled covalently circular DNA, involves intercalation of the phenoxazone ring system between base

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[2] Abbreviations for nucleosides and nucleotides are those recommended by the International Union of Pure and Applied Chemistry-International Union of Biochemistry (IUPAC-IUB) Commission on Biochemical Nomenclature. [*See* Biochemistry 9:4022–4027, 1970.]

[3] Department of Chemistry, University of Rochester, and the Department of Radiation Biology and Biophysics, University of Rochester School of Medicine and Dentistry, Rochester, NY.

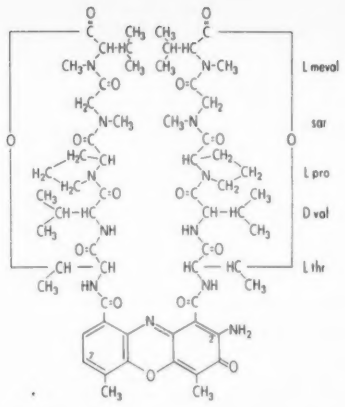

FIGURE 1.—Structure of actinomycin C_1 (D). Meval = *N*-methylvaline; sar = sarcosine; pro = proline; val = valine; thr = threonine.

pairs in the DNA helix analogous to the binding of aminoacridines to DNA (9,13,14). This model, however, leaves unexplained the origin of guanine specificity in the binding reaction (however, *see* Crothers and Müller, pp 97–100, this issue).

We have already described a detailed molecular model for actinomycin-DNA binding that uses the geometry determined by X-ray crystallography of an actinomycin-deoxyguanosine crystalline complex (15–19). The model is of broad interest for several reasons. First, it provides a structural explanation for a large mass of physical and biochemical data concerning the interaction of actinomycin with DNA and this (for the first time) correlates the three-dimensional structure of an antibiotic with its biologic action. Second, the binding of actinomycin to DNA demonstrates two important structural features utilized by DNA in binding other types of molecules. The first feature, intercalation, is used in binding a large variety of drugs and antibiotics to the DNA helix (14) and the model provides detailed stereochemical information concerning this. The second feature concerns the demonstration of a general symmetry principle that is central in our understanding of protein-nucleic acid recognition (20–23). The actinomycin-DNA stereochemical model presented here provides detailed information concerning this important principle.

SOLUTION STUDIES OF THE ACTINOMYCIN-DNA INTERACTION

Much evidence concerning the interaction of actinomycin with DNA is available. It can be summarized as follows.

Evidence Concerning the Polynucleotide Conformation

Actinomycin binds tightly to double-helical DNA, but weakly, if at all, to double-helical RNA, an RNA-DNA hybrid helix, or single-stranded forms of DNA or RNA (24–26).

Evidence Favoring Hydrogen-Bonded Recognition

The binding of actinomycin to DNA requires the presence of deoxyguanosine residues, the 2-amino group on guanine being essential for the binding reaction (4). Thus, synthetic DNA-like polymers of the type $d(A\text{-}T)_n$, $dA_n \cdot dT_n$, $d(I\text{-}C)_n$, and $dI_n \cdot dC_n$ do not bind actinomycin, whereas polymers of the type of $d(G\text{-}C)_n$, $dG_n \cdot dC_n$, $d(DAP\text{-}T)_n$, and $d(2AP\text{-}T)_n$[4] bind actinomycin tightly (7,11,24) (fig 2). The 2-amino group on the purine residue appears to be essential for the binding reaction.

Substitution of the 2-amino group on the phenoxazone ring system with a hydroxyl-,

[4] DAP = 2,6-diaminopurine; 2AP = 2-aminopurine.

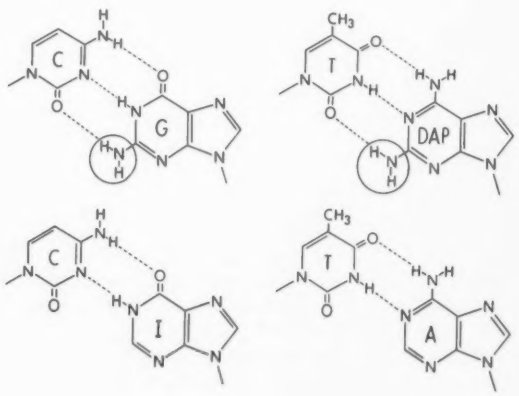

FIGURE 2.—Unit structures of the synthetic polymers used in actinomycin binding studies. DAP = 2,6-diaminopurine; 2AP = 2-aminopurine. The 2-amino group on the purine residue appears to be essential for the actinomycin binding reaction.

chloro-, or dimethylene amino group inhibits the actinomycin-DNA binding reaction (9,12).

Evidence Favoring Intercalation

Addition of actinomycin to solutions containing low molecular weight (10^5 daltons) DNA causes an increase in the intrinsic viscosity and a decrease in the sedimentation constant for the complex (9).

The spectral changes observed when actinomycin binds to DNA can be mimicked by the addition of a large number of different aryl sulfonates to solutions of actinomycin (9).

The kinetics of the association and dissociation of actinomycin from DNA are sensitive to chemical substituents introduced at the 7-position of the phenoxazone ring system (9).

The binding of actinomycin to supercoiled covalently circular DNA causes unwinding of the DNA, as is evidenced by its sedimentation behavior at increasing concentrations of actinomycin (13).

The Actinomycin-Deoxyguanosine Model Reaction

It has been known for many years that a variety of purine nucleosides form complexes with actinomycin resulting in spectral changes similar to those observed in the actinomycin-DNA interaction (8). Of all the purine nucleosides tested, deoxyguanosine complexes most tightly with actinomycin, the association constant being 1.7×10^3 M^{-1} (26,27). If ethanol-water solutions of actinomycin and deoxyguanosine are prepared and allowed to evaporate slowly at room temperature over several days, large single crystals develop. These crystals contain actinomycin and deoxyguanosine co-crystallized as a 1:2 stoichiometric complex (fig 3). The stoichiometry of the solid-state complex appears to be characteristic of the crystalline complex itself and does not reflect the stoichiometry of the solution from which the crystals arise.

FIGURE 3.—Single crystals of the actinomycin-deoxyguanosine crystalline complex. (Approximately × 20)

THE ACTINOMYCIN-DEOXYGUANOSINE CRYSTALLINE COMPLEX

Details of the X-ray analysis are not presented here since they have already been reported (18). The crystalline complex consists of one actinomycin molecule, two deoxyguanosine molecules, and 12 water molecules—a total of 140 atoms (excluding hydrogens) in the asymmetric unit. The structure is observed at atomic resolution.

Actinomycin Has Twofold Symmetry

The twofold symmetry of actinomycin is shown in figure 4 in a computer-drawn illustration of the actinomycin molecule viewed down its approximate dyad axis (which lies roughly along a vector connecting the O–N bridging atoms in the phenoxazone ring). Although the symmetry is not exact (exact symmetry would require the amino group and the quinoid oxygen to be on both sides of the chromophore residue), both polypeptide chains closely obey this noncrystallographic twofold symmetry. The conformations of the peptide linkages are as follows: L-threonine-D-valine, *trans;* D-valine-L-proline, *cis;* L-proline-sarcosine, *cis;* sarcosine-L-*N*-methylvaline, *trans;* L-threonine-carboxamide carbonyl oxygen and carbon of chromophore, *trans.* A strong hydrogen bond exists between neighboring cyclic pentapeptide chains connecting the N–H of one D-valine residue with the carbonyl oxygen of the other D-valine residue (2.94 Å, 2.96 Å). No other hydrogen bonds stabilize the actinomycin structure either between chains or within chains. These findings are consistent with nuclear magnetic resonance data implicating the D-valine N–H groups as hydrogen bond donors. The findings also serve to confirm several of the assignments made regarding the conformation of the polypeptide chains (28–30).

It is evident from figure 4 that the quinoidal portion of the phenoxazone ring system is slightly twisted in a propeller-like fashion with respect to the aromatic portion of the chromophore. A marked deviation from planarity is observed for the amino group nitrogen atom, which lies 0.5 Å from the least-squares plane of the phenoxazone ring system.

The Complex Has Twofold Symmetry

The twofold symmetry of the complex is shown in figure 5. The two deoxyguanosine molecules interact with the two cyclic pentapeptide residues and stack on alternate sides of the phenoxazone ring system. A strong hydrogen bond [2.80 Å, 2.82 Å] connects the guanine 2-amino group with the carbonyl oxygen of the L-threonine residue. A weaker hydrogen bond connects the guanine 3-nitrogen with the N–H group on this same L-threonine residue [3.15 Å, 3.25 Å]. The conformation of both deoxyguanosine molecules is *anti* (this describes the relative orientation of the deoxyribose residues with the guanine rings) (31). One deoxyribose residue is puckered C3′*endo*-C2′*exo*, while the other is C2′*endo*-C3′*exo* (32). The sugar residues of both deoxyguanosine molecules are in close steric juxtaposition with the isopropyl groups of the L-*N*-methylvaline residues (*see* fig 6), and hydrophobic contacts such as these, as well as the stacking of guanine and phenoxazone rings, impart stabilization to the complex. Hydrogen bonding, however, plays a key role in the association; this explains the specificity that actinomycin demonstrates for guanine residues in the model reaction and in the binding reaction with DNA. Related studies of associations between monomer purines and pyrimidines in solution and in the crystalline state have demonstrated the importance of hydrogen bonding in base-pairing recognition (33–35), and, although the details of the stereochemistry differ, the same is true in this example of protein-nucleic acid recognition.

The 1:2 stoichiometry of the complex is a direct consequence of the two-fold symmetry of actinomycin and reflects the two chemically equivalent binding sites available to deoxyguanosine for complex formation. It will be seen that this has deeper meaning with regard to actinomycin-DNA binding, and, perhaps, repressor-operator binding in general.

Water Structure

The complete crystal structure is shown in figure 7. The structure contains 140 atoms in the asymmetric unit; 12 of these atoms are water oxygens (these and several symmetry-

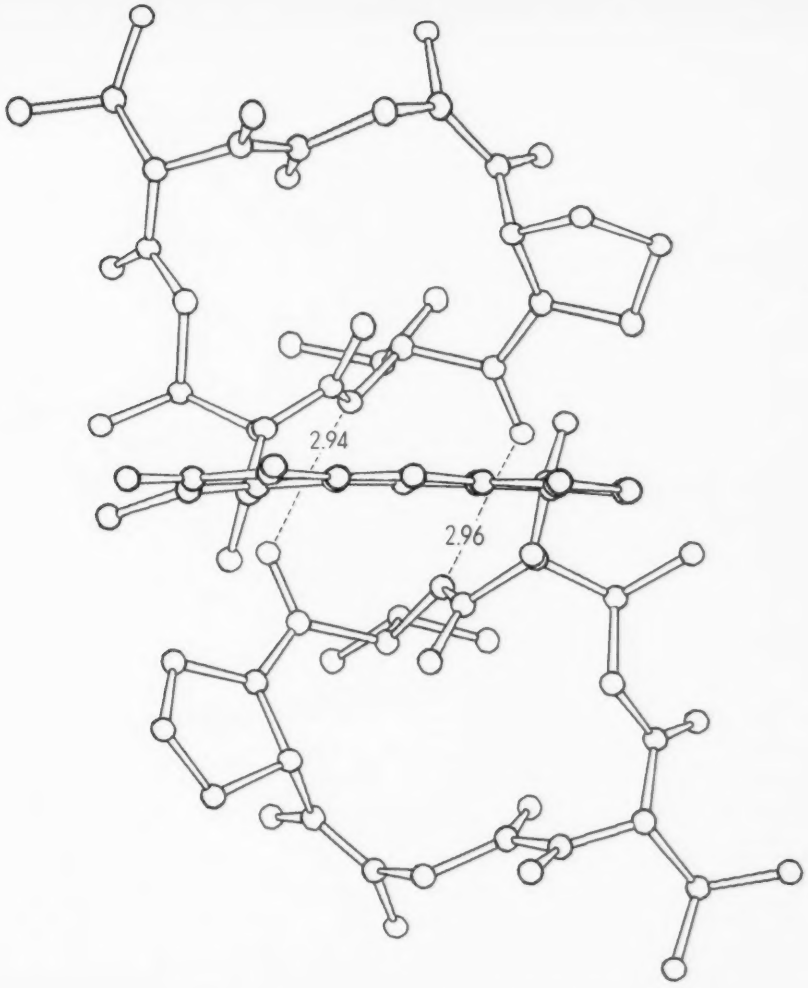

FIGURE 4.—Computer-drawn illustration of the actinomycin molecule viewed down its approximate dyad axis. The phenoxazone ring is in the center of the figure and projects out toward the viewer. Hydrogen bonds connecting D-valine residues on neighboring chains are indicated by dashed lines. Note the twist of the quinoidal portion of the phenoxazone ring system and the nonplanarity of the 2-amino group.

related water oxygens are shown as black balls). The water structure surrounding the complex is of particular interest in view of the large positive entropy change that accompanies actinomycin-DNA binding. This change probably reflects the rearrangement of the water structure during the binding reaction (26). Similar, but smaller, entropy changes accompany the actinomycin-deoxyguanosine solution association.

The structure is stabilized by a total of 152 hydrogen bonds in each unit cell. Approximately 80% of these bonds involve interactions with water structure. Although symmetry has been observed in the complex, there appears to be little or no symmetry in the water structure immediately surrounding the complex. This reflects, in part, the presence of amino and quinoid oxygen groups in the phenoxazone ring system, which are involved in

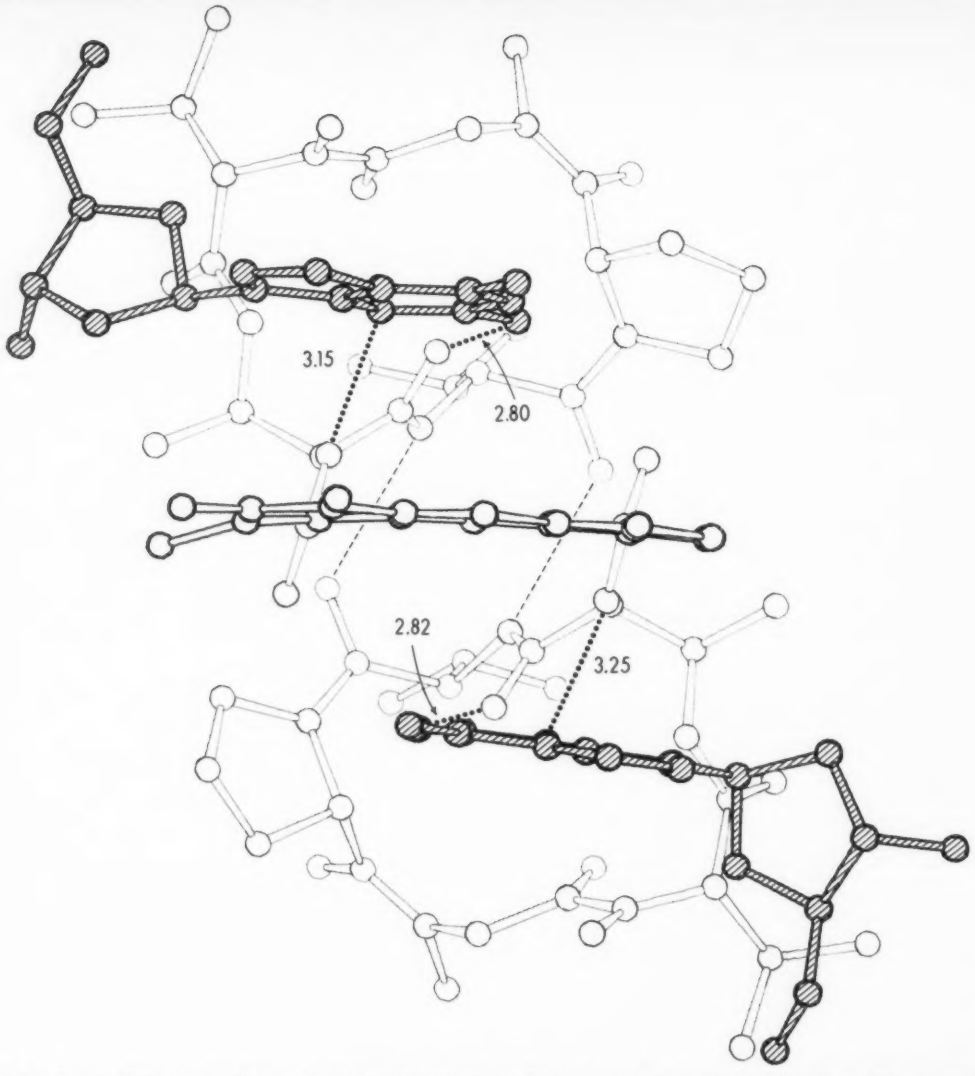

FIGURE 5.—Same view as in figure 4, but with the two deoxyguanosine molecules in place. The approximate twofold symmetry of the complex is apparent. Notice the slight alteration in the stacking of the deoxyguanosine molecule immediately above the quinoidal portion of the phenoxazone ring system resulting from the nonplanarity of the chromophore in this region. Dotted lines indicate hydrogen bonds connecting the actinomycin pentapeptide chains with the deoxyguanosine molecules.

water hydrogen-bonding contacts, as well as a large amount of intermolecular hydrogen bonding between symmetry-related complexes involving water bridges between various functional groups of the nucleosides and peptide chains. A significant number of water-water hydrogen-bonding contacts are observed; some of these result in "ice-like" tetrahedrally coordinated structures. However, most of the contacts act to bridge various functional groups on the actinomycin and deoxyguanosine molecules, both within and between symmetry-related asymmetric units. The water structure appears to be well ordered and rigidly held in this structure (in contrast with the situation in protein crystals); this probably accounts for the hardness and excellent diffraction that these crystals demonstrate.

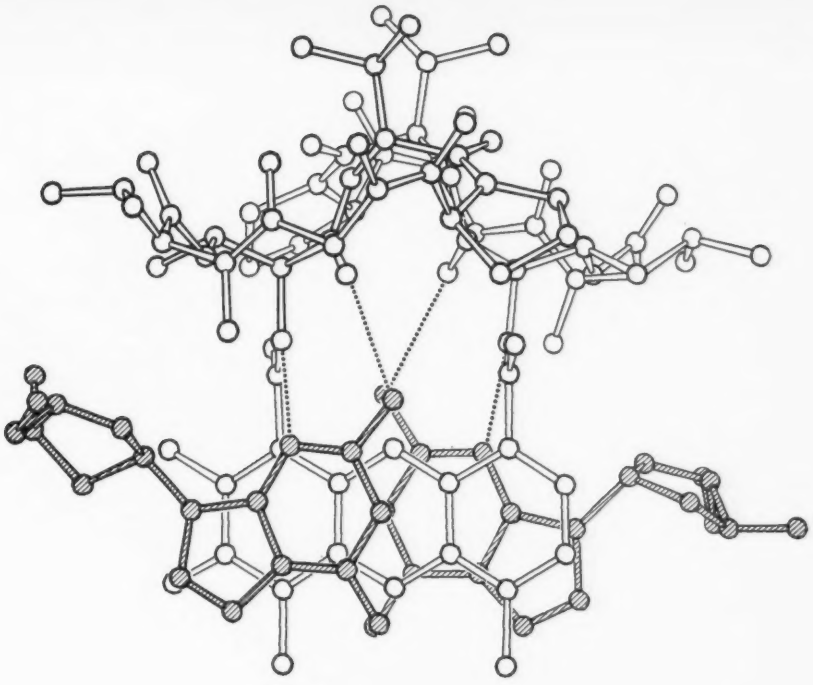

FIGURE 6.—Computer-drawn illustration of the actinomycin-deoxyguanosine complex viewed at an 80° angle to the phenoxazone ring system showing the sandwich-like stacked arrangement of the guanine and phenoxazone rings. Dotted lines indicate hydrogen bonds connecting the actinomycin pentapeptide chains with the deoxyguanosine molecules.

Almost all donor and acceptor groups are involved in hydrogen bonding. Exceptions are the 7-nitrogen on one of the deoxyguanosines and the *N*-methylvaline carbonyl oxygen atom on one of the two pentapeptide chains. Although the stacking of guanine and phenoxazone rings imparts energy of stabilization to the complex, it is clear that hydrogen bonding makes a much larger contribution to the total free energy of the lattice.

THE ACTINOMYCIN-DNA COMPLEX

Intercalation and Hydrogen Bonding to Base-Paired dG-dC Sequences

This section provides a model for actinomycin-DNA binding that uses the geometry determined in the actinomycin-deoxyguanosine crystalline complex (fig 8). Deoxycytidine-5′-phosphate is placed opposite deoxyguanosine to form a hydrogen-bonded guanine-cytosine base pair. Adjacent deoxyguanosine and deoxycytidine residues immediately above and below the chromophore can then be connected by a phosphodiester bridge, giving rise to the sequence dG-dC. An immediate consequence of this is the close steric juxtaposition of the 2-amino group on the chromophore residue and a phosphate oxygen. There is also close steric contact between this amino group and the deoxycytidine furanose ring oxygen. These contacts suggest possible hydrogen bonding between these groups, although, in the case of the phosphate oxygen, hydrogen bonding may utilize a water bridge. Although this amino group appears to be important for actinomycin-DNA binding, it is not essential for the actinomycin-deoxyguanosine solution association (36). Previously, this had suggested a geometric differ-

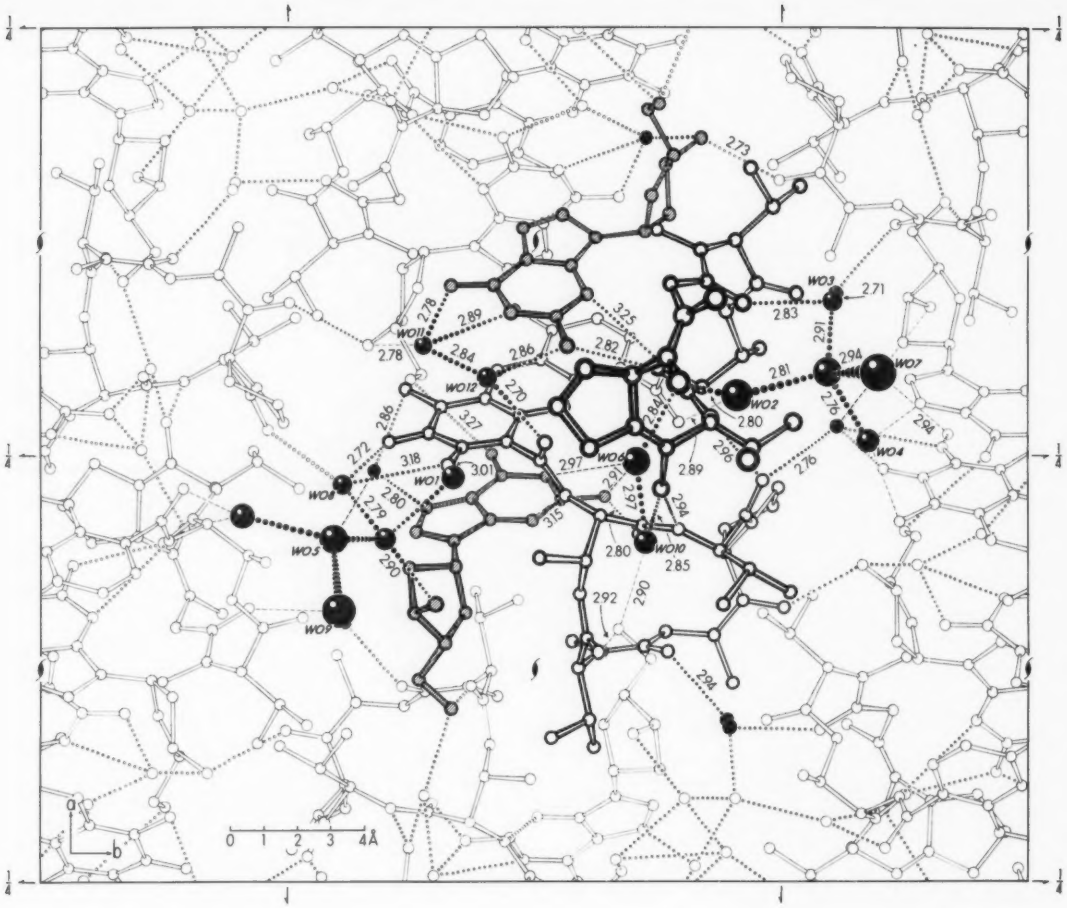

FIGURE 7.—The actinomycin-deoxyguanosine crystal structure as viewed down the *c* axis. Black circles with highlights indicate water molecules. Deoxyguanosine molecules have been shaded with striped lines and are seen stacked above and below the plane of the chromophore residue. The actinomycin molecule is indicated with dark open lines and circles. The surrounding crystal structure is shown with lighter lines. Black dots indicate hydrogen bonds between atoms at neighboring levels. Dashed lines indicate hydrogen bonds between atoms located at different levels in adjoining unit cells. Additional hydrogen bonding in the surrounding structure is indicated by small open circles [From Jain and Sobell (18). Reproduced with permission from the *Journal of Molecular Biology*.]

ence between these associations, but this must now be reexamined. Figures 9–11 show the complex with two base pairs added above and below the central intercalated base-paired dG-dC sequence. The twofold symmetry of the entire complex is apparent. This reflects the twofold symmetry of the hexanucleotide duplex (both the sugar-phosphate chains as well as, in this case, the polynucleotide base sequence) coinciding with the twofold symmetry relating subunits on the actinomycin molecule.

The model predicts, therefore, that the chromophore of actinomycin intercalates between the base-paired dinucleotide sequence dG-dC, while the peptide subunits lie in the narrow groove of the DNA helix and interact with deoxyguanosine residues on opposite chains through specific hydrogen bonds.

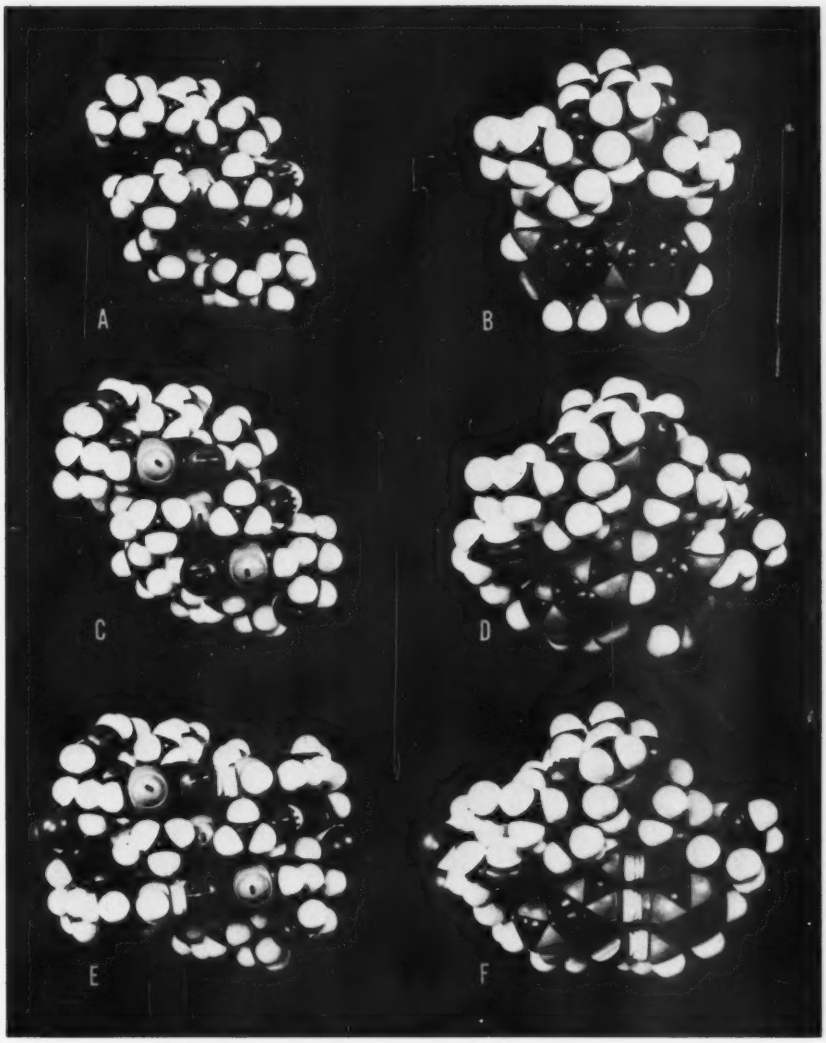

FIGURE 8.—Stepwise assembly of the actinomycin-DNA complex using the space-filling Corey-Pauling-Koltun (CPK) molecular models. Top: *A*. Actinomycin viewed down its dyad axis. *B*. Actinomycin viewed perpendicular to the plane of the chromophore. Middle: *C* and *D*. Same views of the actinomycin-deoxyguanosine complex. Bottom: *E* and *F*. Same views of the actinomycin-[dG-dC] complex. [From Sobell (17). Reproduced with permission from Academic Press, Inc.]

Supporting Evidence

The stereochemical model that we propose reconciles the data suggesting intercalation with the data suggesting hydrogen-bonded recognition, and, in a sense, is a synthesis of the two previous models advanced for actinomycin-DNA binding. The specificity that actinomycin demonstrates in binding double-helical DNA (but not double-helical RNA or an RNA-DNA hybrid helix) most probably reflects the steric requirement imposed by the

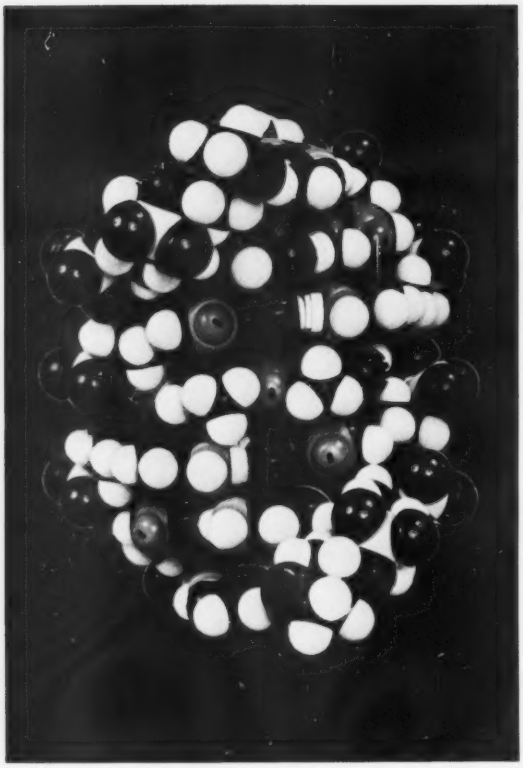

FIGURE 9.—Corey-Pauling-Koltun (CPK) space-filling model of actinomycin complexed with six base pairs viewed from the deep groove of the DNA helix. This model is obtained by adding two base pairs above and two below the central intercalated dG-dC sequence shown in the lower portion of figure 8 (*E* and *F*). The twofold symmetry of this complex is apparent. [From Sobell (17). Reproduced with permission from Academic Press, Inc.]

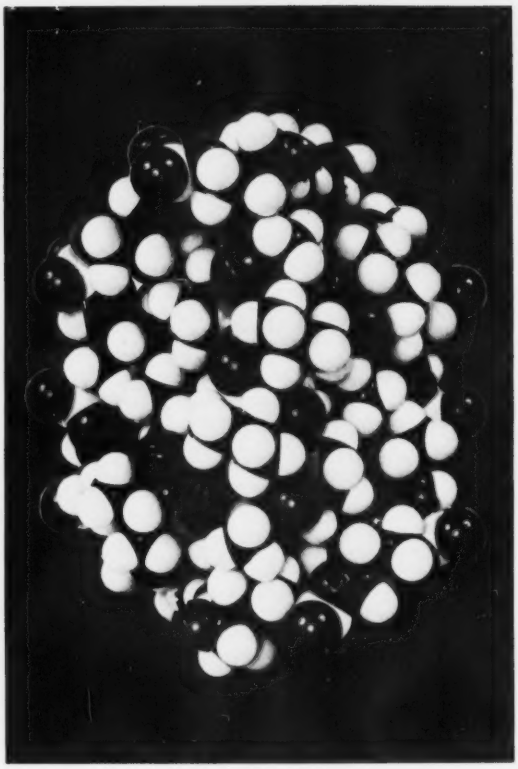

FIGURE 10.—Corey-Pauling-Koltun (CPK) space-filling model of actinomycin complexed with six base pairs viewed from the narrow groove of the DNA helix. The peptide subunits of actinomycin form numerous van der Waals contacts with atoms in both sugar-phosphate chains. The twofold symmetry of this complex is apparent. [From Sobell (17). Reproduced with permission from Academic Press, Inc.]

cyclic pentapeptides to recognize the B rather than the A form of a polynucleotide duplex. The rigidity that double-helical RNA and the RNA-DNA hybrid helix demonstrate with regard to their A conformation may be equally important in excluding actinomycin binding (37–39). The large number of favorable van der Waals contacts made between the cyclic peptides and atoms in the narrow groove of the DNA helix undoubtedly add stability to the complex and act to exclude water during complex formation. Amino acid substitutions that interfere with this favorable fit would reduce the biologic potency of the antibiotic. This is demonstrated in table 1. When proline is replaced by hydroxyproline (or *O*-acetyl hydroxyproline) in the β-pentapeptide ring, the biologic activity of the antibiotic is diminished some twentyfold. However, when proline is replaced by L-*allo*-hydroxyproline (or *O*-acetyl-L-*allo*-hydroxyproline), there is only a small change in biologic activity. This position effect may be readily interpreted from the actinomycin-DNA stereochemical model presented here.

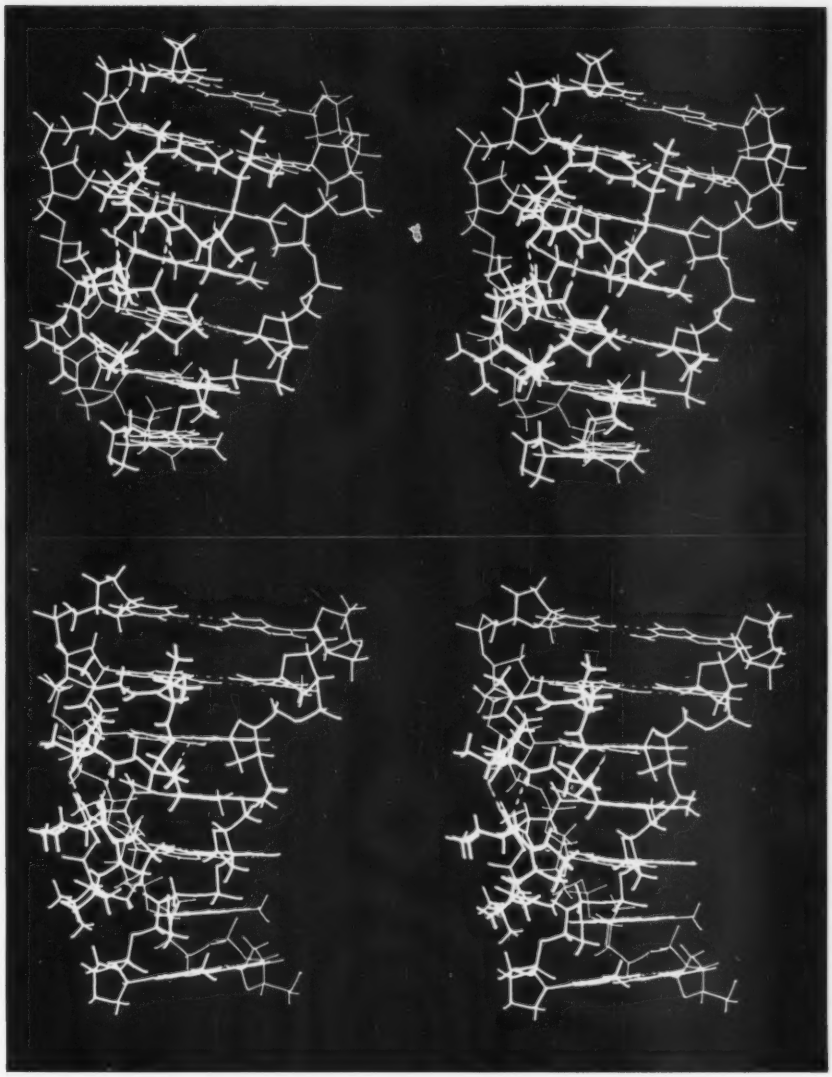

FIGURE 11.—Computer-generated stereo pairs showing the actinomycin-DNA model (kindly supplied by Dr. Louis Katz, Graphic Facility for Interactive Displays, Department of Biological Sciences, Columbia University, New York, NY).

An immediate prediction that this model allows is that poly[d(G-C)] should bind actinomycin most firmly and with the highest efficiency (every 12 nucleotides or every six base pairs) of all the synthetic DNA duplexes. This is verified in table 2. Other sequences containing guanine can bind actinomycin, but they do so with lower affinity and with less efficiency. This can be explained by assuming a complex which has similar geometry but which forms fewer (less than four) hydrogen bonds between the cyclic pentapeptides and the dinucleotide base-paired sequence. Steric strain may accompany this altered interaction, and

TABLE 1.—Effect of selected single amino acid substitutions on the antibacterial activity of actinomycin*

Actinomycin	Relative activity		Amino acid residue† in position 3 of—	
			β Chain	α Chain
C_1	100		L-Pro	L-Pro
X_2	215	200	L-γ-oxoPro	L-Pro
$X_{0\delta}$	57	50	L-aHyp	L-Pro
O-Acetyl $X_{0\delta}$		25	L-aHyp(OAc)	L-Pro
$X_{0\beta}$	2.5	5	L-Hyp	L-Pro
O-Acetyl $X_{0\beta}$		1.5	L-Hyp(OAc)	L-Pro
X_{1a}	100		L-γ-oxoPro	Sar

*Adapted from Reich and Goldberg (6).

†Pro = proline; aHyp = L-*allo*-4-hydroxyproline; Hyp = L-4-hydroxyproline; sar = sarcosine.

TABLE 2.—Equilibrium constants and number of binding sites for DNA-actinomycin interactions*

DNA	G + C content (%)	K_{app} (M^{-1}) × 10^{-6}	Nucleotides per site
Micrococcus luteus DNA	72	2.5	9
Salmon sperm DNA	42	2.0	20
Poly[d(G-C)]	100	3.2	12
Poly(dG)·poly(dC)	100	2.0	91
Poly[d(T-G)]·poly[d(C-A)]	50	1.3	25
Poly[d(T-C)]·poly[d(G-A)]	50	0.6	37
Poly[d(T-T-G)]·poly[d(C-A-A)]	33	1.2	45
Poly[d(T-T-C)]·poly[d(G-A-A)]	33	0.7	67
Poly[d(T-A-C)]·poly[d(G-T-A)]	33	0.8	50
Poly(dI)	—	1.3	111

*Adapted from Wells and Larson (7).

this could explain the diminished efficiency of the binding observed with these polymers. Related data on the interaction between actinomycin and a large number of deoxyribodinucleotide monophosphates are now available (40). The dinucleotide pdG-dC interacts strongly with actinomycin and in a cooperative fashion (*see* fig 12). Other sequences (either individually or as complementary pairs) bind actinomycin less tightly and do not demonstrate cooperativity. It is of interest that the four dinucleotides pdC-dG, pdT-dG, pdA-dG, and pdG-dG (in the presence or in the absence of the complementary sequence) resemble deoxyguanosine in their binding properties, the reaction being distinctly first order in character. Dinucleotides of the type pdG-dT and pdG-dA bind actinomycin less tightly, with the former binding actinomycin more tightly than the latter. Again, the reaction appears to possess first-order characteristics.

These observations can be explained in stereochemical terms as follows(40): In the case of dinucleotides with the general sequence pdN-dG (where N represents either thymidine, cytidine, adenosine, or guanosine), bind-

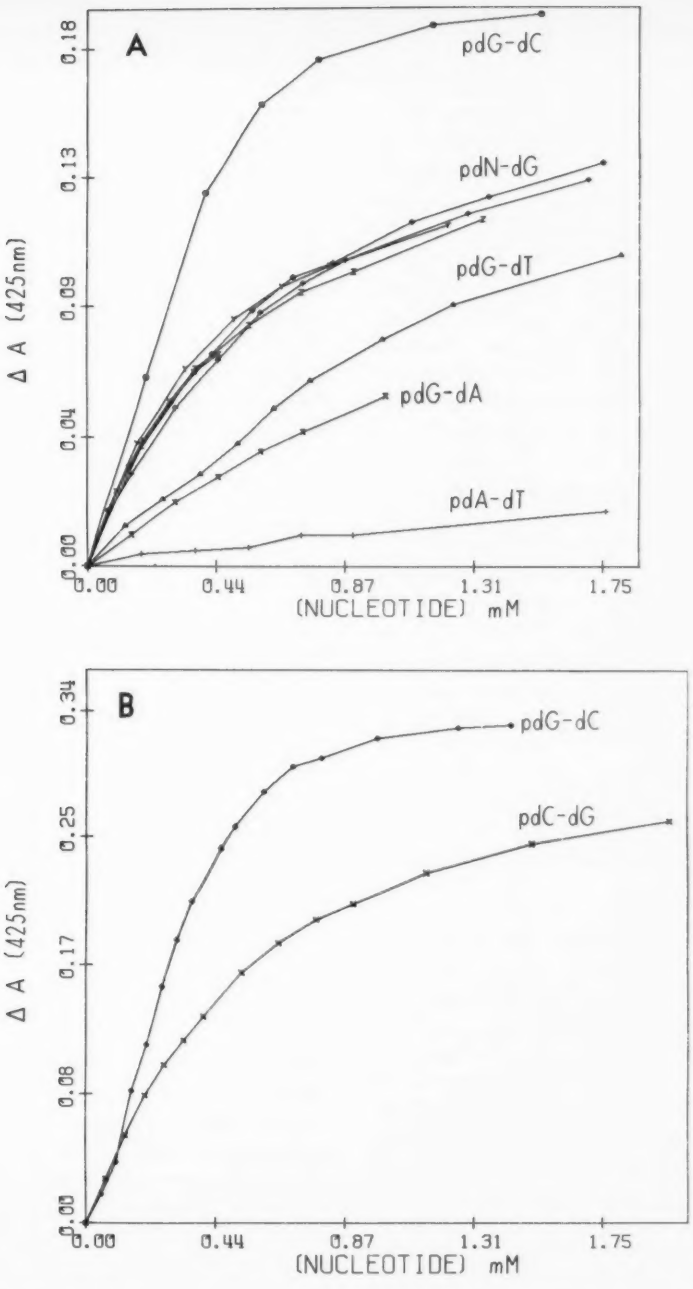

FIGURE 12.—*A.* Actinomycin-deoxyribodinucleotide binding studies, as monitored by difference spectra at 425 nm. N corresponds to thymidine, cytidine, adenosine, or guanosine. Actinomycin concentration = 20 μmol (μM). *B.* Actinomycin-[pdG-dC] and actinomycin-[pdC-dG] binding curves. Actinomycin concentration = 36 μmol. [From Krugh (40). Reproduced with permission from the *Proceedings of the National Academy of Sciences* and T. R. Krugh.]

ing occurs in the same hydrogen-bonded geometry found in the actinomycin-deoxyguanosine crystalline complex. This involves interaction with both cyclic peptide chains independently without steric interference. These complexes would not involve intercalation. Dinucleotides of the type pdG-dA and pdG-dT can use the same hydrogen-bonded configuration as in the crystalline complex; however, these complexes would involve intercalation. (These observations follow immediately from an examination of Corey-Pauling-Koltun [CPK] models of these complexes.) Here, complex formation between one dinucleotide and actinomycin would interfere with further complex formation with the second dinucleotide. This reflects the altered stereochemistry imposed by a noncomplementary dG-dT or dG-dA base pair. In the first case (guanine-thymine base pairing), the fit resembles the Watson-Crick type more closely than in the second case (guanine-adenine base pairing). The interference effect almost certainly reflects stereochemical considerations such as these.

Detailed kinetic studies on the binding of actinomycin with DNA demonstrate that several fast steps occur initially in the binding reaction, followed by a slow step extending over several minutes (9). This slow step in binding has also been observed in actinomycin binding studies with pdG-dC (40). Although it is not possible to directly relate these kinetic steps to specific stereochemical events accompanying binding, it has been suggested that the prolonged last step may reflect a conformational change affecting the peptide subunits in the actinomycin molecule (9). An alternative explanation, however, is that an additional conformational change occurs in the polynucleotide backbone during the final stages of complex formation; this involves a change in the deoxyribose sugar puckering from C2′*endo*-C3′*exo* (as found in the B form of DNA) to C3′*endo*-C2′*exo* (as observed in the final detailed model). The detailed actinomycin-DNA stereochemical model proposed involves no significant conformational change in the actinomycin pentapeptides (other than, perhaps, a change in the puckering of one of two proline rings); for this reason, we favor the latter explanation at this time. Further information is necessary to establish this point.

Therefore, one can envisage the stepwise association of actinomycin with DNA as follows: Actinomycin first intercalates between a base-paired dG-dC sequence, with recognition being initially provided by the specific water structure surrounding actinomycin and the polynucleotide backbone and base sequence. As intercalation continues, this ordered water structure is "squeezed out," and specific hydrogen bonding with guanine residues takes place. The polynucleotide conformation at this stage may exist with all deoxyribose sugars puckered C2′*endo*-C3′*exo*, with unwinding of the helix reflecting the altered stereochemistry in the immediate intercalation site (−18°). The final slow step is an alteration of the deoxyguanosine sugar puckering to C3′*endo*-C2′*exo*, which provides a better steric fit between the actinomycin cyclic peptides and the sugar-phosphate chain. This last step adds stability and specificity to the binding reaction and it is accompanied by additional helix unwinding of base pairs immediately above and below the intercalated base-paired dG-dC sequence (−16°).

POSSIBLE MEDICAL IMPLICATIONS

The detailed stereochemistry of actinomycin binding to DNA suggests a rationale for the synthesis of new variants of the actinomycin molecule that may be used clinically in the chemotherapy for neoplastic disease and viral infection. One of the major problems in using actinomycin clinically is its extreme toxicity since it passes through the cellular membrane of both normal and malignant cells without selectivity. If the peptide portion of the actinomycin molecule could be altered so as to change the permeability properties of the molecule with respect to normal and malignant cells, the antibiotic could become clinically useful. Thus, for example, D-valine could be replaced by a derivative of D-valine possessing a long alkyl chain, giving the actinomycin molecule a "tail." A substitution such as this probably would not affect the ability of the molecule to bind to DNA, but it might selectively prevent entrance of the antibiotic into

a given type of cell. One would hope that it would be possible to devise a "tail" that would prevent the antibiotic from passing through the cellular membrane of normal cells but which would have little effect on transport across the cellular membrane of malignant cells. Similarly, one might covalently attach the actinomycin molecule (through its "tail") to serum albumin and hope that the malignant cells that demonstrate pronounced pinocytosis would phagocytize these molecules and poison themselves with actinomycin. Other possible modifications could involve stereospecific peptide subunits designed to recognize other types of base sequences in DNA. These would serve as useful probes in the study of nucleic acid function and could perhaps be used in the treatment of certain viral (DNA) infections. Finally, it may be possible to create an actinomycin-like antibiotic that is able to bind to double-helical RNA. This would perhaps be useful in the treatment of viral (RNA) infections as well as in oncogenic research.

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Summation and General Discussion

Seymour Perry [1]

Actinomycin D (NSC–3053) was the first antibiotic for which antitumor activity was demonstrated in the early 1950s in Germany. In this country, Dr. Farber studied its activity against experimental tumors in mice and reported his results in a small abstract (1). He then demonstrated its clinical usefulness in suppressing Wilms' tumor in children (2). Subsequently, enhancement of the drug's activity by radiotherapy has been reported, and it has been shown capable of curing Wilms' tumor when combined with surgery and radiotherapy. By now, the usefulness of actinomycin D has been clearly established in Wilms' tumor (when combined with radiotherapy) and in gestational choriocarcinoma in women. It also has significant activity in testicular tumors, embryonal rhabdomyosarcomas, lymphomas, and, perhaps, Ewing's sarcomas. It is interesting to note that these activities were recognized years ago, but unfortunately the early trials in other tumors were often inadequate, generally involving small numbers of patients, and the response rates were low. However, there is a recent report by the Children's Cancer Study Group A suggesting that actinomycin D might be useful for maintenance of response in acute leukemia (3).

At the basic level, particularly in the chemistry of the actinomycins, we have heard about the vast amount of information which is available on the synthesis and biosynthesis of actinomycin D and its analogs. A great deal is also known about the chemical-physical characteristics of these compounds. However, there is still much to be learned, eg, cell uptake of actinomycin; solubility and partition characteristics; pharmacology and pharmacokinetics of actinomycins; practical problems of production, separation, purity, and formulation; the best route of administration; metabolism in animals or man; and the mode of action and the activity of these materials in relation to growth characteristics of tumors. Cross resistance has not yet been studied. Finally, very little is known about the use of actinomycin D in combination with other drugs and other modalities of therapy.

Dr. Goldin has presented the available data on preclinical testing and screening of the actinomycins, but since these data were acquired years ago, many of the studies were undoubtedly done with mixtures of compounds or with impure materials which renders interpretation of the results very difficult. We know virtually nothing about structure-activity relationships of actinomycins in these test systems. The possibilities of testing analogs appear virtually unlimited, but evaluation of many of the analogs will be limited because such small amounts of material are available. In addition to all the known natural materials, there are approximately 100 synthetics and derivatives of the natural products. Unfortunately, most of these are not presently available and if preclinical tests are to be undertaken, they must be limited to representative prototype compounds which can be prepared so that structure-activity relationships can be evaluated. Dr. Goldin has estimated that perhaps gram amounts would be needed for fully comprehensive testing, but an order of magnitude less than that might be sufficient for evaluation in the new econo-screen.

From a broader aspect, comparative studies with related compounds (the anthracycline antibiotics, adriamycin [NSC-123127] and daunorubicin [NSC-83142]) would be very important. This group of compounds is, of course, of great interest in the clinic at present. Adriamycin is of particular importance since it has the widest spectrum of clinical activity of any known compound. An intense effort to increase our fundamental knowledge concerning all of these materials would seem to be imperative. A thorough understanding of all aspects of the

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mechanism of action of actinomycin is needed, and certainly studies on the molecular level are vital. This, in turn, might lead to the design of more active and more specific compounds.

There is another compelling reason why the actinomycins should be the objects of greater study. At present, there are about 25–30 antitumor compounds with known clinical usefulness. It is important to note that only a few of these can effect cures when used singly: cyclophosphamide in Burkitt's tumor; methotrexate in choriocarcinoma; and actinomycin D in Wilms' tumor.[2] In light of this, it is apparent that the actinomycins need to be investigated to a far greater extent than has been the case in the past. I have already discussed the need for more studies at the basic and preclinical levels and I have mentioned the apparently inadequate clinical studies which have been done in the past. It is also obvious that additional clinical studies should be undertaken, although it is often difficult to stimulate clinical interest in an old agent. Certainly, the effort should be made.

Summary of General Discussion

After the summation a general discussion was opened and speakers were called upon to present their views on the potential of actinomycins for cancer chemotherapy and how these potentials might be best exploited and implicated. The main points of this discussion will be given in summarized form without attempting to identify individual discussants.

It was pointed out that a relatively small structural variation between daunorubicin (R = H) and adriamycin (R = OH) (fig 1) makes an enormous difference in the spectrum of antitumor activities both in experimental systems and in man. In virtually all of the experimental tumor studies adriamycin has a therapeutic index superior to daunorubicin at the optimal LD10 dose; against L1210 leukemia in mice daunorubicin is inactive, whereas adriamycin exhibits considerable activity. In clinical trials

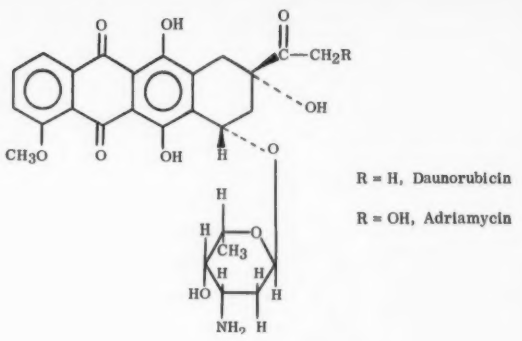

FIGURE 1.—Basic chemical structure for adriamycin and daunorubicin.

in lymphomas adriamycin is one of the most active agents (daunorubicin is minimally active). It elicits responses in 40%–60% of patients with metastatic breast cancer (daunorubicin has a 12% response rate), and it shows a promising 30%–40% reaction against sarcomas in adults and children. These results demonstrate (*a*) that compounds exerting a molecular mechanism of action similar to that of actinomycin D (ie, interaction with DNA and inhibition of DNA-dependent RNA synthesis) exhibit a relatively broad range of antitumor activity in man, and (*b*) that a slight difference in structure can cause a very significant improvement in the therapeutic index. This makes it very important to investigate carefully some of the analogs of actinomycin D with the modern techniques now available.

Two actinomycins, D (C_1) and C_3, presently being used in cancer chemotherapy in man,[3] differ considerably in lipophilicity, ie, solubility in organic solvents. The suggestion was made to carry out a clinical study comparing the therapeutic efficacy of these two actinomycins, eg, in the treatment of Wilms' tumor in children or gestational choriocarcinoma in women. It was pointed out that statistically significant studies of this nature would require large numbers of patients not normally available even in

[2] Cyclophosphamide: NSC-26271; CAS reg. No. 6055-19-2; 2*H*-1,3,2-oxazaphosphorine, 2-[bis(2-chloroethyl)-amino]tetrahydro-, 2-oxide, monohydrate.

Methotrexate: NSC-740; CAS reg. No. 59-05-2; glutamic acid, *N*-[*p*-[[(2,4-diamino-6-pteridinyl)methyl]methylamino]benzoyl]-.

[3] Actinomycin D used in the United States: Cosmegen, produced by Merck Sharp and Dohme, Rahway, NJ. Actinomycin C_3 used in Europe: major component of Sanamycin, produced by Farbenfabriken Bayer, AG, Wuppertal-Elberfeld, Germany.

the major cancer centers because of the relatively infrequent occurrence of these tumors. Instead, phase I clinical studies should be done with various selected actinomycin analogs.

The discussion then turned to the problem of deciding which analogs should be chosen for both experimental and clinical investigations. As with several other classes of therapeutic agents, structural permutations can be myriad, and indeed, about 100 actinomycin analogs and derivatives have been described. It was pointed out, however, that very few analogs are actually available in amounts sufficient for in vivo screening. It was suggested that the chemists propose a limited number of representative prototypes which appear to be promising for resynthesis or scale-up synthesis to allow in vivo testing in experimental systems.

A lively discussion arose on the development of a satisfactory experimental screen for actinomycin analogs. It will be important to include a system in addition to our standard screen (L1210 leukemia). The Lewis lung carcinoma was suggested as part of a minimal screen which could also consist of P388 leukemia, B16 melanoma (to test for increased analog effect), and Ridgway osteogenic sarcoma (to ensure no decreased effect), as well as the L1210 leukemia.

The value of tissue culture systems for preliminary screening was discussed, since considerably smaller amounts of drug are required. Attention was called to the high predictive value of an in vitro system using *Escherichia coli* DNA-dependent RNA polymerase for testing activities of actinomycin analogs. Correlations of inhibitory capacity in this test, which requires minimal amounts of compounds, with bacteriostatic potency and antitumor activity in some experimental systems have been very good for the analogs tested. This test could, of course, be very useful for quickly eliminating negative compounds, ie, analogs or derivatives that are totally devoid of any inhibitory activity.

The development of new compounds might be pursued by the chemist in three ways: (*a*) biosynthesis of peptide analogs; (*b*) preparation of derivatives starting from actinomycin D, actinomycin C_3, or actinomycin X_2; and (*c*) total chemical synthesis of peptide or chromophore analogs. The first two approaches are attractive from a practical point of view in providing a reasonable amount of material via a few reaction steps. Actinomycins containing pipecolic acid in place of proline have been prepared biosynthetically and await screening. It was pointed out that several 7-substituted derivatives are highly active, and that 7-aminoactinomycin D, although of little bacteriostatic potency, showed antitumor activity against Ridgway osteogenic sarcoma in mice to a degree comparable with actinomycin D. Finally, total synthesis of actinomycin analogs offers considerably broader possibilities of structural variation, albeit at the expense of much larger efforts and time requirements.

The chemists would greatly welcome a type of integrated rationale for the design of new clinically useful actinomycin analogs or derivatives—a rationale that would be based on (*a*) comparative clinical pharmacologic and pharmacokinetic properties, (*b*) comparative experimental antitumor spectrum and bacteriostatic effects, (*c*) toxicity and physiologic distribution parameters, (*d*) uptake and retention in tissues and tumors versus sensitivity, (*e*) solubility and, especially, comparative lipophilic versus hydrophilic behavior, (*f*) kinetics of interaction with DNA and binding constants, (*g*) rate of inhibition of RNA synthesis and effects on protein biosynthesis, and (*h*) conformation through nuclear magnetic resonance spectroscopy and X-ray crystallographic structure analysis. Although the design of the synthetic analogs and derivatives reported in the past was based on certain rational considerations, it has hardly been possible to verify their validity. This was so because after all the laborious synthetic work, the compounds underwent only antibacterial testing and very little or no experimental antitumor screening or molecular biologic studies. An appeal was made, therefore, to initiate a collaborative interdisciplinary and interinstitutional approach to determine the above-mentioned properties and parameters in a defined, statistically valid, and comparable form for all those actinomycins that should prove to be of clinical usefulness. These data will then allow to base rationales for the design of

new analogs on those drug parameters that appear to be important for attaining improved cancer chemotherapeutic properties.

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Closing Remarks

Emil Frei III [1]

As a result of this Conference on Actinomycins, I would propose the following:

1. Re-testing of Actinomycins

The available actinomycin D (NSC-3053) analogs and prototypes of synthetic derivatives should be re-evaluated in transplanted tumor systems using modern techniques. There are perhaps eight or ten such compounds that are available in sufficient quantity for re-testing. The re-testing should be performed in (*a*) L1210 leukemia, the standard primary screen; (*b*) P388 leukemia, a tumor system sensitive to actinomycin D and to natural products generally; (*c*) the B16 or S91 melanomas, slow-growing solid tumors which are sensitive to actinomycin D; and (*d*) Lewis lung tumor, a slow-growing solid tumor which is insensitive to actinomycin D. These studies may provide leads to further studies on structure-activity correlations both with respect to organic synthesis and to in vitro and in vivo experimental and possible clinical screening.

2. Clinical Pharmacology

Although actinomycin D has been employed clinically for 17 years, there is no information on its clinical pharmacology. The drug has been used at a rather fixed dose schedule. Pharmacologic studies in experimental systems suggest that uptake and retention by cells correlate with selective tissue toxicity. Clinical pharmacologic studies should be performed with both pulse treatment and continuous infusion in an effort to define the plasma half-time; protein binding; distribution; uptake by red cells, lymphocytes, granulocytes, and, where possible, tumor; biotransformation; and excretion. Such studies will aid in the clinical application of this agent and, perhaps more importantly, will serve as a frame of reference for the evaluation of analogs whose advantages might relate to differing pharmacology.

Finally, in vitro uptake studies of human tissues might be undertaken to determine whether the correlation between uptake and response, as Schwartz et al (1) and Kessel and Wodinsky (2) have defined, also pertains to man. If it does, a predictive system for man will have been developed.

3. Further Therapeutic Trials

On the basis of clinical review, further therapeutic trials of actinomycin D are indicated. This is perhaps particularly true of melanoma and lymphoma. Also, differing dose schedules and, in particular, large single intermittent doses should be used in phase I clinical trials.

In order to implement the above, I suggest that a small working group be established.

I would like to thank all of our speakers, particularly the visiting speakers from this country and, perhaps most particularly, our visitor from overseas, Dr. Hans Brockmann, who played such a major pioneering role in developing the actinomycins. I would also like to thank Dr. Johannes Meienhofer, who was the major organizer of this meeting, and our friends from the National Cancer Institute, Drs. Seymour Perry and Abraham Goldin, for their scientific participation and support. Finally, I would like to thank Dr. Sidney Farber, who provided the initial pioneering clinical impetus for this study. His continuing interest and support has had much to do with the development of this conference.

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